University of Alberta

Ribonuclease and cytokinin modulating activities of Pathogenesis-related (PR) 10 proteins in plant responses to abiotic stresses

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Plant Science

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta
Fall 2006

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Your file Votre référence
ISBN: 978-0-494-23112-8
Our file Notre référence
ISBN: 978-0-494-23112-8

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ABSTRACT

Abiotic stresses such as salinity, drought and temperature extremes cause extensive loss to agricultural production worldwide. In order to further characterize plant responses to salinity, a proteomics-based investigation to identify and validate proteins whose levels were altered by salinity in pea (*Pisum sativum* L.) roots was initiated. Several proteins including pathogenesis-related (PR) 10 proteins were identified using this approach. In order to test the hypothesis that PR 10 proteins play a crucial role in mediating plant responses to stress, we characterized transgenic *Brassica napus* and *Arabidopsis thaliana* plants expressing two different pea PR 10 cDNAs, *PR 10.1* and *ABR17*, respectively. Enhanced germination, early seedling growth and tolerance to abiotic stresses were observed in these transgenic plants. PR 10 proteins from various species have been demonstrated to be RNases, the RNase activity of pea PR 10 proteins was demonstrated by overexpressing the cDNAs of *PR 10.1* and *ABR17* in *Escherichia coli* and assaying for RNase activity. Since tRNA (and rRNA) can be sources of cytokinins (CKs) in plants, it was hypothesized that the PR 10-mediated effects were due to CK levels being modulated by the expression of PR 10 cDNAs in transgenic plants. Increased levels of CK in both transgenic *B. napus* and *A. thaliana* were observed which paralleled elevated expression of the primary CK-response regulator, *ARR5*, in *A. thaliana*. A role for the elevated CKs in mediating enhanced germination and early seedling growth of transgenic plants was confirmed by exogenous application of CK which increased germination and growth of wild type *A. thaliana*. Results presented in this study have demonstrated a crucial role for *PR 10* genes in abiotic stress responses of plants.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my academic supervisor Dr. Nat Kav for his valuable guidance, constructive suggestions, encouragements and support throughout the course of my doctoral research. His enthusiasm, close observations, dynamic personality and tireless efforts as a mentor are truly appreciated. I feel highly privileged in expressing my profound sense of gratitude for my supervisory committee members Drs. John Hoddinott and Mike Deyholos for their invaluable suggestions and insightful criticism to review my dissertation. I am indebted to Drs. Saleh Shah, Hafiz Rahman, Stephen Strelkov, Laki Goonewardene, Randall Weselake, Mohan Thiagarajah, Neil Emery, David Reid, Leonid Kurepin and Brian Fristensky who provided their invaluable guidance and expertise in my research.

I gratefully acknowledge the technical help and kind cooperation provided by William Yajima and other fellow graduate students Mohsen Mohammadi, Nidhi Sharma, Kelley Dunfield, Bo Yang, Yue Liang, Sowmya Krishaswamy, Aparna Prasad and Debangshu Bhaumick. Their invaluable suggestions, timely support and helpful attitude created a very friendly ambience during my research. I wish to express my thanks to Lorne Burke and Paul Semchuk at the Institute for Biomolecular Design (IBD), University of Alberta for their assistance with Mass Spectrometry. I would also like to acknowledge the timely help and constant encouragement exemplified by Dr. Krish Dhunnoo and my friends Kamlesh Raiter, Vijaya Raghavan and Veeramani. Their continuous support has given me a big boost to enhance my morale and keeping the momentum up throughout the course of my research.

Finally, I would like to thank my parents and family members. Though far away, they did not allow their absence to come in the way of helping and supporting me. Even during very tough moments when my family was shattered with the untimely demise of my ten year old niece "angel" Gargi from brain tumor, they gave their warmth and support so that I can concentrate on my research. No words and thanks can be enough for their constant encouragement, profound patience and immense faith in me without which I would not have achieved my goals.

Financial support from the University of Alberta is gratefully acknowledged.

Dedicated to my niece: "angel" Gargi

&

Silent worship of my parents

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABR17	ABA-responsive proteins (ABR) 17
ABF	ABRE-binding factor
ABRE	Abscisic acid responsive element
ACE	ACGT-containing element
ANOVA	Analysis of variance
AREB	ABA-responsive-element-binding protein
ARR	*Arabidopsis* response regulator
BAP	Benzyl amino purine
BSA	Bovine serum albumin
bZIP	Basic-domain leucine-zipper
CaM	Calmodulin
CDPK	Ca^{2+} dependent protein kinases
CK	Cytokinin
COR	Cold regulated
CRT	C-repeat
CSBP	Cytokinin-specific binding proteins
DAG	Diacylglycerol
DEAE	Diethyl amino ethyl
DHN	Dehydrin
DRE	Drought responsive element
DREB	DRE-binding factor

DTT	Dithiothreitol
EREBP	Ethylene-responsive element binding protein
ERF	Ethylene-responsive-element-binding factor
ESI	Electrospray Ionization
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
GA	Gibberellic acid
GLM	General linear model
GPX	Glutathione peroxidase
GR-RBP	Glycine-rich RNA-binding proteins
GST	Glutathione S-transferase
HOS1	High expression of osmotic responsive 1
HSP	Heat shock proteins
IAA	Indole acetic acid
ICE1	Inducer of CBF expression 1
IFR	Isoflavone reductase
Ins-5-Pase	Inositol polyphosphate 5-phosphate
IP3	Inositol 1,4,5-triphsophate
IPR	Intracellular PR
IPT	Isopentenyl transferase
JA	Jasmonic acid
KIN	Cold-inducible
LEA	Late embryogenesis abundant

MAPK	Mitogen-activated protein kinases
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MBF1c	Multi protein bridging factor 1c
MEP	Methylerythritol phosphate
MLP	Major latex proteins
MS	Mass spectrometer
MVA	Mevalonate
MYBRS	MYB recognition sequence
MYCRS	MYC recognition sequence
NDPK	Nucleoside diphosphate kinase
NORC	Nonselective outward-rectifying conductance
NPK	*Nicotiana* protein kinase
NR	Nitrate Reductase
NSC	Nonselective cation channels
NTA	Nitrilotriacetic acid
OEE	Oxygen evolving enhancer
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PIP2	Phosphatidylinositol 4-5-bisphosphate
PLC	Phospholipase, phospholipase C
PLD	Phospholipase D
PR	Pathogenesis-related

PR 10	Pathogenesis-related (PR) 10
ProDH	Proline dehydrogenase
PVDF	Polyvinylidene fluoride
RCA	Rubisco activase
RD	Responsive to desiccation
RNase	Ribonuclease
ROS	Reactive oxygen species
RWC	Relative water content
SDS	Sodium dodecyl sulphate
SIMK	Salt stress inducible MAPK
SNF	Sucrose non-fermenting kinases
SOD	Superoxide dismutase
SOS	Salt overly sensitive
TBP	Tributylphosphine
TCA	Trichloroacetic acid
TOF	Time of flight
TPS	Trehalose-6-phosphate (T-6-P) synthase
VIC	Voltage-independent cation channels

1. General Introduction

1.1. Agriculture and food production

The practice of agriculture first began around 8000 BC and, ever since, one of the major challenges has been to provide food for the increasing human population. Total world population is currently at 6.6 billion and is expected to rise to nearly 9.1 billion by 2050 posing additional challenges on our capabilities to produce food (http://www.un.org/esa/population/). Possible ways to increase food production includes increasing arable land and/or yield (http://www.fao.org/docrep/). Increasing arable land, however, may not be a viable option due to decreasing area and the marginalization of the available land. For example, a loss of 30% of all arable land by 2025 and up to 50% by the middle of 21st century due to increased salinization is expected (Wang *et al.*, 2003) which leaves increasing crop yield as the only viable alternative to address the challenges of a growing world population.

The "green revolution" of the 1960s pioneered by Dr. Norman Borlaug was responsible for dramatic increases in yield brought about by the development of high-yielding, hybrid varieties and technological inputs. However, the regular yield increases enjoyed over the past 50 years have now reached a plateu, and once again we are faced with the challenge of having to increase our agricultural yield (Yamaguchi and Blumwald, 2005). Conventional breeding strategies have met with only limited success in developing abiotic stress tolerant plants. However, the advent of molecular techniques and information from genome sequencing projects during the last decade have rapidly advanced our understanding of the genetic and biochemical basis of abiotic stress tolerance, and accelerated the generation of transgenic plants that exhibiting tolerance to various abiotic stresses (Apse *et al.*, 1999; Yamaguchi and Blumwald, 2005). In order to ensure food security for an increasing world population, generation of novel, stress-tolerant crop varieties will be crucial, and a multivariate approach based on conventional plant breeding and modern genetic engineering approaches will be required (Flowers, 2004; Yamaguchi and Blumwald, 2005).

1.2. Salinity stress

Soil salinity is a major environmental stress affecting agricultural productivity worldwide (Boyer, 1982). In addition to the 25% arid and desert land (dry land salinity) of our planet, 20% of the irrigated land (irrigated land salinity) is also affected by salinity stress (Yamaguchi and Blumwald, 2005). It is now estimated that more than 800 million hectares of land throughout the world are affected by salinity (FAO, 2005). Many factors such as evaporation precipitation and weathering of rock affect salt concentration (Mahajan and Tuteja, 2005). At present, approximately 10% of agricultural land in Western Australia is affected by dryland salinity and in the future this area is expected to double due to saline groundwater discharge and associated salt load (Taylor and Hoxley, 2003). Salt stress is a major problem on the Canadian prairies and a linear increase in the size and severity of salt-affected areas has been reported. For example, saline seeps in the northwestern Great Plains regions are increasing at a rate of 5% per year (http://www1.agric.gov.ab.ca/). Drought also occurs very frequently in Alberta (especially southern Alberta) and during 2000-2001 Alberta faced one of the most severe droughts during the last 78 years (http://www1.agric.gov.ab.ca/). Heavily irrigated agricultural lands are highly saline since drier agricultural land needs intense irrigation and due to evapotranspiration, salt delivered along with the irrigation water becomes concentrated in the soil (Mahajan and Tuteja, 2005).

1.2.1. Effects of salt stress on plant cells

Salinity causes both hyperionic and hyperosmotic stress in plants (Hasegawa *et al.*, 2000). The ionic component of stress increases the Na^+ and Cl^- toxicity and alters the K^+/Na^+ ratios, whereas the osmotic component affects plant water uptake by decreasing total soil water potential (Hasegawa *et al.*, 2000). Water potential consists of pressure potential and osmotic potential, and as a result of increased salt concentrations, leaf water potential and osmotic potential decreases, whereas leaf turgor pressure increases to balance the water requirement of the whole plant (Morales *et al.*, 1998; Hernandez *et al.*, 1999). Significant changes in water potentials can impose osmotic stress which disrupts the cellular activities and ultimately may cause plant death (Xiong and Zhu, 2002). Therefore, the overall effects of salt stress are three-fold: reduction of water potential, disturbances in ion homeostasis and increased ion toxicity (Parida and Das, 2000). Salt stress reduces growth leading to a stunted appearance (Hernandez *et al.*, 1995), decreases fresh and dry weight of leaves, stem and roots (Hernandez *et al.*, 1995; Chartzoolakis and Klapaki, 2000), increases the thickness of the epidermal and mesophyll layers as well as the diameter of palisade and spongy cells (Longstreth and Nobel, 1979), and reduces the chlorophyll and total carotenoid contents of leaves (Hernandez *et al.*, 1995; 1999). The exact nature of the spectrum of damages inflicted by high salt concentrations is not entirely clear. However, in general salinity affects all major plant physiological processes including the activities of various enzymes, photosynthesis, protein synthesis, membrane disorganization and nutrient acquisition. The detrimental effects of salt results in a range of symptoms such as chlorosis and necrosis. Furthermore, when the capacity of the cell to store salts is exhausted, cell dehydration and the death results (Kurniadie and Redmann, 1999; Hasegawa *et al.*, 2000; Xu *et al.*, 2000).

1.2.2. Sodium transport in plant cells

1.2.2.1. Na^+ influx

Under normal physiological conditions, plants maintain a high K^+/Na^+ ratio in the cytosol but an influx of Na^+ results in accumulation of Na^+ ions and alteration of cytosolic K^+/Na^+ ratio (Blumwald *et al.*, 2000). Na^+ initially enters cells of the root epidermis and cortex from the soil solution in an unidirectional Na^+ influx which is a key determinant of overall shoot Na^+ accumulation (Schubert and Lauchli, 1990; Davenport and Tester, 2000) and is distinct from net influx, which is the end result of both influx and efflux (Essah *et al.*, 2003). The negative membrane potential of the plasma membrane favors the passive transport of Na^+ which enters the plant cells through high-affinity K^+ transporters (HKT1; Rus *et al.*, 2001), non-selective cation channels, such as nonselective outward-rectifying conductance (NORC), and voltage-independent cation channels (VIC) (Roberts and Tester, 1997; Tyerman *et al.*, 1997; Wegner and deBoer, 1997; Amtmann and Sanders, 1999) or Na^+ leakage into the root via the apoplast (Yeo *et al.*, 1999). However, the relative contribution of each of these pathways varies with species and growth conditions (Tester and Davenport, 2003; Zhu, 2003). The plant 'Salt Overly Sensitive' (SOS)3 gene encodes a protein that contains a Ca^{2+}-binding domain and it has been demonstrated that Na^+-influx activity of the high-affinity transporter *A. thaliana* HKT1 (AtHKT1) is negatively regulated by the SOS pathway (Zhu, 2003).

1.2.2.2. Na^+ efflux

Plant cells transport Na^+ against the electrochemical potential gradient by an active process that is mediated by plasma membrane H^+-ATPase (Sussman, 1994). H^+-ATPase uses the energy of ATP hydrolysis and generates a proton motive force to allow the function of a plasma membrane Na^+/H^+ antiporter which couples the efflux of Na^+ with the inward movement of H^+ (Blumwald *et al.*, 2000). It has been demonstrated that levels of transcripts of H^+-ATPases increase due to salt stress (Niu *et al.*, 1993). Na^+/H^+ antiporters are encoded by the *SOS1* gene which coordinates between transpirational Na^+ flow and the vacuolar sequestration of Na^+ in leaves (Zhu 2003). The overexpression of *SOS1* in *A. thaliana* enhanced salt tolerance (Shi *et al.*, 2003), and the overexpression of H^+-pyrophosphatase, *AVP1* (Gaxiola *et al.*, 2001) enhances salt and drought tolerance.

1.2.2.3. Na^+ compartmentation

The vacuolar sequestration of Na^+ by lowering Na^+ concentration in the cytoplasm and osmotic adjustment provides an efficient mechanism to prevent some of the deleterious effects of Na^+ (Zhu, 2003). It has been demonstrated that the overexpression of *AtNHX1*, a vacuolar Na^+/H^+ antiporter in various plants, enhances salt tolerance (Apse *et al.*, 1999; Ohta *et al.*, 2002). Although the precise mechanisms that regulate the activity of the vacuolar Na^+/H^+ antiporters during normal and stress conditions are not clear, it has been suggested that the vacuolar membrane Na^+/H^+ exchange activity in *A. thaliana* is regulated by the SOS pathway (Qiu *et al.*, 2002).

1.3. Mechanism of salt tolerance

1.3.1. Complexity of salt tolerance

The generation of salt-tolerant crops has been a quest of plant breeders and varietal differences as well as intraspecific selection have been evaluated for salt tolerance (Epstein *et al.*, 1980). The first attempt to evaluate the inheritance of salt tolerance by an interspecific cross of *Lycopersicon esculentum* (*Lem.*) and *Lycopersicon pimpinellifolium* (*Jusl.*) was made by Lyon (1941); however, the scarcity of new salt-tolerant varieties has limited the success of traditional breeding approaches (Flowers and Yeo, 1995). Physiological, as well as genetic, studies support the view that salt tolerance is a complex trait and its sub-traits might be determined by multiple gene loci (Flowers, 2004). However, increased salinity tolerance conferred by the constitutive overexpression of specific genes (Apse *et al.*, 1999; Gaxiola *et al.*, 2001; Zhang and Blumwald, 2001; Luo *et al.*, 2005) has demonstrated that manipulation of cellular processes by genetic engineering may be a viable approach for enhancing plant-tolerance to salinity stress (Tester and Davenport, 2003).

1.3.2. Aspects of plant salt tolerance

1.3.2.1. Homeostasis

As described earlier, salinity disrupts ion homeostasis at both cellular- and whole-plant levels and one of the major aspects of plant tolerance to salt stress is based on their ability to re-establish this homeostasis during high salinity. Na^+ influx, vacuolar compartmentation and Na^+ contribute to the lower Na^+ concentrations in cytoplasm (Hasegawa *et al.*, 2000; Zhu, 2001[1]). Involvement of many ATPases, water channel proteins and ion transporters that are regulated by salt stress is crucial for ion homeostasis (Xiong and Zhu, 2001). Na^+ influx is controlled by various ion transporters such as nonselective cation channels (NSCs), HKT1 and LCT1. Therefore, investigation of the function of transporters in preventing the Na^+ influx is very crucial for enhancing salt tolerance (Tester and Davenport, 2003; Zhu, 2003).

Na^+ compartmentation is another way of reducing Na^+ toxicity in the cytosol. Many salinity-tolerant plants use stored Na^+ as an osmolyte in the vacuole to establish osmotic homeostasis (Flowers *et al.*, 1977). Osmolytes or compatible solutes function in osmotic adjustment by lowering cellular osmotic potential to facilitate water absorption and restore intracellular salt concentrations (Yancey *et al.*, 1982). Compatible solutes can also act as free-radical scavengers or chemical chaperones and directly stabilize cell membranes and proteins (Hare *et al.*, 1998; Diamant *et al.*, 2001). For example, molecules such as proline and glycine-betaine have received significant attention with respect to their roles in maintaining osmotic homeostasis under salinity stress (Delauney and Verma, 1993; Chen and Murata, 2002). Transgenic studies have also demonstrated that increasing cellular concentrations of proline and glycine-betaine can increase the tolerance of plants to salinity stress.

In higher plant cells, Na^+ ions are extruded from the cells or compartmentalized in their vacuoles mainly by Na^+/H^+ antiporters. Unlike animal, fungal or algal cells, plant cells do not appear to possess the Na^+-ATPases for Na^+ efflux; however, they possess a Na^+/H^+ antiporter (Blumwald *et al.*, 2000) which removes Na^+ from the cells or compartmentalizes it in their vacuoles. Plasma membrane and tonoplast localized antiporters use a pH gradient generated by P-type H^+-ATPase and V-type H^+-ATPases or H^+-pyrophosphatases (PPase), respectively (Xiong and Zhu, 2002). The overexpression of a vacuolar Na^+/H^+ antiporter in *Arabidopsis* confers salt tolerance and demonstrates the importance of vacuolar transporters for plant salt tolerance (Apse *et al.*, 1999). The overexpression of *SOS1* gene, which encodes a plasma membrane Na^+/H^+ antiporter also enhances salt tolerance in *Arabidopsis* and demonstrates that salt tolerance could be achieved by limiting Na^+ accumulation in plant cells (Shi *et al.*, 2003). Such studies provide a crucial insight into ion homeostasis processes and their regulation.

1.3.2.2. Detoxification of reactive oxygen species (ROS)

Salt stress as well as ionic and water stresses impose oxidative stress via the action of excess reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and superoxide anions. In response to such oxidative stress, plants produce stress-induced proteins, antioxidant compounds and ROS scavenging enzymes to detoxify the ROS (Xiong and Zhu, 2002; Apel and Hirt, 2004). In addition, as mentioned previously, the osmolytes that accumulate in plant cells in response to the osmotic stress imposed by salinity also possess free radical scavenging properties. ROS scavenging by osmolytes and stress-induced proteins is an effective way of coping with the deleterious effects of oxidative stress and such detoxification strategies have been utilized in generating many plants tolerant to salt stresses (Sakamoto *et al.*, 1998). For example, the overexpression of superoxide dismutase (SOD), which catalyses the conversion of superoxide anion to hydrogen peroxides and water enhances tolerance to abiotic stresses such as salinity (Bohnert and Sheveleva, 1998). Transgenic tobacco plants overexpressing a cell wall peroxidase (Amaya *et al.*, 1999) and glutathione S-transferase (Roxas *et al.*, 1997) have higher tolerance to salt stress, whereas transgenic plants with reduced catalase activity exhibit sensitivity to salt stress (Willekens *et al.*, 1997). Studies of transgenic plants with altered levels of several enzymes involved in free radical scavenging further support an important role played by cellular antioxidant defense systems in protecting plants from salinity stress.

1.3.2.3. Growth regulation

The ability of plant cells to recover, maintain and improve the activity of physiological processes that are sensitive to salt is important for their tolerance to such stress (Xiong and Zhu, 2002). Salt stress inhibits plant growth and one reason for a reduction in growth rate is inadequate photosynthesis due to stomatal closure, which reduces uptake of carbon dioxide (Zhu, 2001[a]). Plant growth may also be reduced due to an inhibition of cell division, which was suggested based on the observation that a cyclin dependent-protein-kinase inhibitor ICK1 was up-regulated by abscisic acid (ABA; Wang *et al.*, 1998). Indeed, during salt stress, the cellular concentrations of ABA increases which presumably induces the expression of ICK1, leading to decreased cell division as a result of inhibition of cyclin-dependent protein kinase (Zhu, 2001[a]). Inhibition of cell expansion during salt stress is also possible as a result of lower concentrations of the phytohormones auxin, cytokinin, gibberellins and other growth promoting substances such as brassinosteroids (Zhu, 2001[a]; Xiong and Zhu, 2002).

1.3.3. Perception of salt stress: the SOS pathway

During salinity stress the levels of cytosolic Ca^{2+}, that are transported from the apoplast and intracellular compartments, increase, and this transient increase initiates stress signal transduction (Knight *et al.*, 1997). During salt stress Ca^{2+} signaling is linked to the SOS pathway which helps to re-establish cellular ion homeostasis by extruding excess Na^+ ions via the plasma membrane Na^+/H^+ antiporter (Zhu, 2002). *SOS* mutants were identified by Liu and Zhu (1998) through positional cloning and *SOS* genes (*SOS1*, *SOS2* and *SOS3*) were genetically confirmed to function in a common pathway of salt tolerance (Zhu *et al.*, 1998).

The *SOS3* gene encodes a Ca^{2+} binding protein with Ca^{2+} binding motifs and a myristoylation sequence (MGXXXST/K) at the N-terminus of the protein (Liu and Zhu, 1998; Ishitani *et al.*, 2000). Mutations in the *SOS3* gene reduces its Ca^{2+} binding ability and impairs the ionic equilibrium, with the plants becoming sensitive to salt stress (Liu and Zhu, 1998). Interestingly, this defect was partially restored by the addition of exogenous Ca^{2+} in the medium which suggested that the mutations reduced the affinity of SOS3 for Ca^{2+} (Liu and Zhu, 1998). In response to the increased Ca^{2+}, even with a lower affinity, SOS3 binds Ca^{2+}, interacts with an effector kinase and transduces downstream signals (Zhu, 2002).

SOS2 encodes a novel serine/threonine protein kinase that has an N terminal domain with homology to sucrose non-fermenting kinases (SNF), and a C terminal regulatory domain that possesses a 21 amino acid FISL/NAF motif (Albrecht *et al.*, 2001). In response to salt stress, the SOS2 mRNA was up-regulated in *Arabidopsis*

(Liu *et al.*, 2000). SOS3 interacts with SOS2 via the FISL motif, which is an autoinhibitory domain, and activates its kinase activity in a calcium-dependent manner (Halfter *et al.*, 2000). When *Arabidopsis* double mutants (*SOS3*/*SOS2*) were tested for salt sensitivity, additive effects were not observed suggesting that *SOS3* and *SOS2* function in the same pathway (Halfter *et al.*, 2000). Furthermore, the constitutive over-expression of *SOS2* rescued the salt-sensitive phenotype of both *SOS2* and *SOS3* mutants, providing further evidence that both *SOS3* and *SOS2* function in the same pathway (Zhu, 2002; Chinnusamy *et al.*, 2004). The *SOS1* gene encodes a 127-kDa protein with an N-terminal region of 12 transmembrane domains and has sequence homology with the plasma membrane Na^+/H^+ antiporter and a C-terminal region with a long hydrophilic cytoplasmic tail, which was identified as the target of the SOS3-SOS2 pathway (Shi *et al.*, 2000; Zhu, 2002).

Interaction of the SOS pathway with other proteins such as AtHKT1 which is a low affinity Na^+ transporter that mediates the Na^+ entry into the root cells of *Arabidopsis* during high salt stress, has also been reported (Uozumi *et al.*, 2000). A mutation in AtHKT1 suppresses the *SOS3* mutation, suggesting that the SOS3–SOS2 complex negatively regulates the activity of *AtHKT1* during salt stress, and prevents Na^+ entry into the cell (Rus *et al.*, 2001; Zhu, 2002). The *AtNHX1* gene encodes a tonoplast Na^+/H^+ antiporter that functions in compartmentalizing the excess Na^+ into the vacuole (Apse *et al.*, 1999). Interaction of *SOS2* with a CAX1 (H^+/Ca^{2+}) antiporter has also been demonstrated where activation of CAX1 via SOS2 assists in the maintenance of Ca^{2+} homeostasis (Cheng *et al.*, 2004). From these observations it is clear that the *SOS* gene products play crucial roles in modulating plant responses to salt stress through complex interactions with other proteins involved in maintaining salt homeostasis in plant cells.

1.4. Osmotic stress signaling pathways

1.4.1. Plant responses to osmotic stresses

Plant cells adjust their water potential as per requirements of the whole and significant changes in water potential may lead to osmotic stress in plants (Xiong and Zhu, 2002). Osmotic stress appears to be a common consequence of exposure to salinity, drought and low temperature stresses and the secondary stress signals such as ABA, ROS and phospholipids are also triggered in response to it (Hasegawa *et al.*, 2000). High salinity and drought conditions are the main causes of osmotic stress in plants, which disrupts normal cellular activity and results in a wide range of responses at the molecular, cellular and whole-plant level, and ultimately may lead to plant death (Hasegawa *et al.*, 2000; Xiong and Zhu, 2002). Salt-induced osmotic stress changes include adjustment in ion transport and metabolic changes, whereas survival during freezing-induced osmotic stress may depend on prevention of ice nuclei formation (Chinnusamy *et al.*, 2004). During osmotic stress many changes in gene expression occur and it is possible that multiple signal transduction pathways interact at various steps in individual pathways (Chinnusamy *et al.*, 2004).

1.4.2. Sensing systems

1.4.2.1. Kinase sensors

Our knowledge of stress sensors and their role in abiotic stress cross-talk is still not clear. Osmotic stress may be sensed by transmembrane protein kinases such as two-component histidine kinases (Urao *et al.*, 2000) and wall associated kinases (Kohorn *et al.*, 2001). The two-component system consists of a sensory histidine kinase and a response regulator, functions as a stress sensor. For example, the transmembrane two component histidine kinase, HIK 33 in a cyanobacterium functions a thermosensor (Suzuki *et al.*, 2000) whereas SLN1 in yeast and AtHK1 in *Arabidopsis* function as osmosensors (Maeda *et al.*, 1994). Na^+ may be sensed by a membrane receptor and the Na^+/H^+ plasma membrane antiporter, SOS1, is a possible Na^+ sensor and functioning as both a sensor and transporter of Na^+ (Zhu, 2003).

1.4.2.2. Calcium sensors

As articulated above, Ca^{2+} plays an important role in several physiological processes and regulates them through its action on effector proteins such as calmodulin (CaM), Ca^{2+}-dependent protein kinases (CDPKs) and Ca^{2+} regulated phosphatases (Knight and Knight, 2001).

1.4.2.2.1. Calmodulin (CaM)

CaM is involved in many biological responses during plant growth and development, as well as in response to various stress conditions. CaM contributes to the sensing of Ca^{2+} signals under various stress conditions (Bouche *et al.*, 2005). *CaM* genes are activated in response to salinity stress in tomato (Delumeau *et al.*, 2000), mung bean (Botella and Arteca, 1994). In tobacco a Ca^{2+}CaM-binding kinase (NtCBK2) is induced by high salt (Hua *et al.*, 2003); and the CaM-binding protein (CaMBP), AtCaMBP25, is induced by salinity, cold, mannitol and dehydration (Perruc *et al.*, 2004). CaM is most likely a regulatory component during salinity and osmotic stress as well as being associated with signaling pathways responding to various other environmental stresses (Bouche *et al.*, 2005). *ACA4* encodes a vacuolar Ca^{2+}-ATPase containing CaM-binding proteins in *Arabidopsis* and yeast mutants expressing a full-length or a truncated form of *ACA4* demonstrate tolerance to osmotic stresses (Geisler *et al.*, 2000).

1.4.2.2.2. Ca^{2+} dependent protein kinases (CDPKs)

CDPKs are calmodulin-independent serine/threonine kinases that play crucial roles in stress- and Ca^{2+}-mediated signal transduction (Stone and Walker 1995; Poovaiah *et al.*, 1996; Sheen, 1996). A large family of CDPKs has been implicated in signaling pathways that respond to various abiotic stresses. Induction of OsCDPK7 by cold or salt stress in rice (Saijo *et al.*, 2000) and implication of AtCDPK1 and

AtCDPK2 isoforms of *Arabidopsis* in salt and drought stress suggested that individual CDPK isoforms are involved in specific signalling pathways (Urao *et al.*, 1994; Sheen, 1996). The CDPK substrate protein 1 (CSP1) in the common ice plant (*Mesembryanthemum crystallinum*) phosphorylates CSP1 in a Ca^{2+}-dependent manner and salt stress induces the co-localization of McCDPK1 and CSP1 in the nucleus of ice plants providing further evidence for the role of CDPKs in signaling events following salt stress (Patharkar and Cushman, 2000).

1.4.2.2.3. Ca^{2+} regulated phosphatases

Serine/threonine phosphatases (PPases) are a group of proteins that interact with Ca^{2+} to effect an end response (Luan, 1998) and it includes calcium sensor proteins such as calcineurin B (Kudla *et al.*, 1999). SOS3, encodes a Ca^{2+} binding protein, has significant similarity to the regulatory subunit of yeast calcineurin, and is involved specifically in salt stress tolerance (Liu and Zhu, 1998). Cold-induced inactivation of protein phosphatase is controlled by Ca^{2+} influx in alfalfa (Monroy, 1998). The ABI1 and ABI2 proteins are homologous to type-2C PPases, and have been suggested as negative regulators in the early stages of ABA signal transduction (Sheen, 1998; Gosti *et al.*, 1999). These studies suggest that calcium, a secondary messenger during abiotic stress signalling, regulates kinases and phosphatases involved in the signal transduction and may serve as nodes at which cross-talk can occur (Knight, 2000; Knight and Knight, 2001; Chinusamy *et al.*, 2004).

1.4.3. MAPK cascades

In plants, mitogen-activated protein kinases (MAPKs) are activated by hormones, abiotic and biotic stresses (Tena *et al.*, 2001). The phosphorylation cascade of mitogen-activated protein kinases typically consists of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK) (Knight and Knight, 2001). MAPKs transfer information from sensors to cellular responses and play a very important role in signal transduction pathways (Tena *et al.*, 2001; Nakagami *et al.*, 2005). Activation of MAPKs in response to various abiotic stresses has been reported: in alfalfa, activation of osmo-sensing protein kinase SIMK (salt stress inducible MAPK) in response to hyperosmotic stress (Munnik *et al.*, 1999); in tobacco, activation of SIPK (salicylic acid-induced protein kinase) by hyperosmotic stress (Mikolajczyk *et al.*, 2000); in *Arabidopsis*, activation of AtMPK3 in response to salt stress (Mizoguchi *et al.*, 1996) and AtMPK4 and AtMPK6 in response to salt, cold and low humidity (Ichimura *et al.*, 2000), and in rice, activation of OsMAPK5 in response to abscisic acid and various biotic and abiotic stresses (Xiong and Yang, 2003) have been reported. Activation of the MAPK cascade by reactive oxygen species (ROS) has also been demonstrated; it includes activation of ANP1 (ANP1; *Arabidopsis Nicotiana* protein kinase (NPK)1-like protein kinase; Kovtun *et al.*, 2000) and AtMPK3, AtMPK6 and its positive regulator, NDPK2 (nucleoside diphosphate kinase 2; Moon *et al.*, 2003) by H_2O_2. MAP kinase phosphatases (MKPs) are the negative regulators of MAPKs. *Arabidopsis* MKP1 plants exhibit increased resistance to salinity and microarray analysis indicated the negative regulation of an Na^+/H^+

antiporter (Ulm *et al.*, 2002). Similarly many others studies have also demonstrated involvement of the MAPK cascades in various abiotic and biotic stresses, suggesting a very crucial role of MAPK in cross-talk during abiotic stress signaling (Chinnusamy *et al.*, 2004).

Overexpression of the rice *OsMAPK5* gene (using the 35S cauliflower mosaic virus promoter) increases tolerance to drought, salt, and cold stresses and its suppression (using double-stranded RNA interference; dsRNAi) enhances resistance to fungal (*Magnaporthe grisea*) and bacterial (*Burkholderia glumae*) pathogens and reduces abiotic stress tolerance (Xiong and Yang, 2003). Suppression of *OsMAPK5* expression also results in constitutive expression of pathogenesis-related (PR) genes, such as *PR 1* and *PR 10*, suggesting that *OsMAPK5* can negatively modulate the expression of PR genes and positively regulate abiotic stress tolerance (Xiong and Yang, 2003).

1.4.4. ABA and osmotic stress signalling

ABA is a crucial phytohormone that regulates many aspects of plant development such as seed development, synthesis of seed storage proteins and lipids, seed dormancy and desiccation tolerance of seeds (Finkelstein *et al.*, 2002). In addition to the broad role of ABA in plant growth and development, it also plays a critical role in response to various abiotic stresses such as regulation of water balance and osmotic stress tolerance and therefore, it is also known as a stress hormone (Shinozaki and Yamaguchi-Shinozaki, 2000). During salt and drought stress, ABA controls guard cells and induces genes that encode dehydration tolerance (Finkelstein *et al.*, 2002; Zhu, 2002). Various abiotic stresses result in cell desiccation as well as osmotic imbalance, and the overlap in expression pattern of stress genes after various abiotic stresses suggests that ABA and various abiotic stress signals share common signaling pathways during cross-talk (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein *et al.*, 2002).

There are at least two pathways, ABA-dependent and ABA-independent, that regulate stress-responsive genes during osmotic stress (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein *et al.*, 2002). Although genetic analysis demonstrates that there is no clear demarcation between ABA-dependent and –independent pathways, it has been reported that ABA-dependent signalling pathways play a more crucial role in stress-responsive gene expression during osmotic stress (Xiong *et al.*, 2002). Many genes such as *RD29A*, *RD22*, *COR15A; COR47* and *P5CS* are regulated by ABA during osmotic stresses (Xiong *et al.*, 2001).

1.4.5. Transcription factors

In the past few years significant progress has been made in understanding the transcriptional regulation of some stress responsive genes. Many stress-responsive genes contain *cis*-elements such as the drought responsive element (DRE), the abscisic acid responsive element (ABRE) and the MYC recognition sequence (MYCRS), and MYB recognition sequence (MYBRS) in their promoters (Leung and Giraudat, 1998; Zhu, 2002). Many cold stress regulated genes contain DRE/C-repeat (CRT) *cis*-

DRE/C-repeat (CRT) *cis*-element in their promoter which has the core sequence CCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger, *et al.*, 1997).

The ABA-dependent pathway activates basic leucine zipper transcription factors such as ABRE-binding protein (AREBs)/ABRE-binding factors (ABFs) which, in turn, induce stress-responsive genes (Figure 1-1; Xiong *et al.*, 2002). Expression studies of *RD29A* suggested that its promoter contains both ABRE and DRE/CRT elements and that it can be activated by ABA-dependent and independent pathways (Narusake *et al.*, 2000). A negative regulator of cold-induced CBF transcription was identified as high expression of osmotic responsive 1 (*HOS1*; Ishitani *et al.*, 1998). *HOS1* encodes a novel protein that contains a RING-finger motif, which may function in the degradation of CBF, DREB1 and in the induction of CBF expression 1 (ICE1) proteins (Lee *et al.*, 2001). The *HOS1* and *HOS2* loci encode signaling components and regulate gene expression only in response to ABA and osmotic stresses (Ishitani *et al.*, 1998; Lee *et al.*, 1999).

Figure 1-1. Transcriptional regulatory networks of *cis*-acting elements and transcription factors involved in stress-responsive gene expression. Various transcription factors triggered in response to abiotic stresses (top) are shown in ellipses (middle) and *cis*-acting elements involved in stress-responsive transcription are shown in boxes (bottom). Salinity and drought activates DREB2A, DREB2B, bZIP and MYB/MYC transcription factors which interact with CRT/DRE, ABRE or MYBRE/MYCRE elements in the promoter of stress genes. Cold temperature activates the transcription of CBF/DREB1 family of transcriptional activators. CBF/DREB1 proteins bind to DRE/CRT *cis*-elements in promoters of *RD*/*COR*/*LT1*/*KIN* genes and activate the expression of these genes. *HOS1* functions as a negative regulator of ICE1. ABA-dependent abiotic stress signalling is mediated in part through IP3 and Ca^{2+}. *FRY1* functions as a negative regulator of IP3 during drought, cold and ABA-responses and *ABI* functions as a negative regulator for ABA signalling (Zhu, 2001[b]; Xiong *et al.*, 2002; Zhu, 2002; Shinozaki *et al.*, 2003; Mahajan and Tuteja, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006).

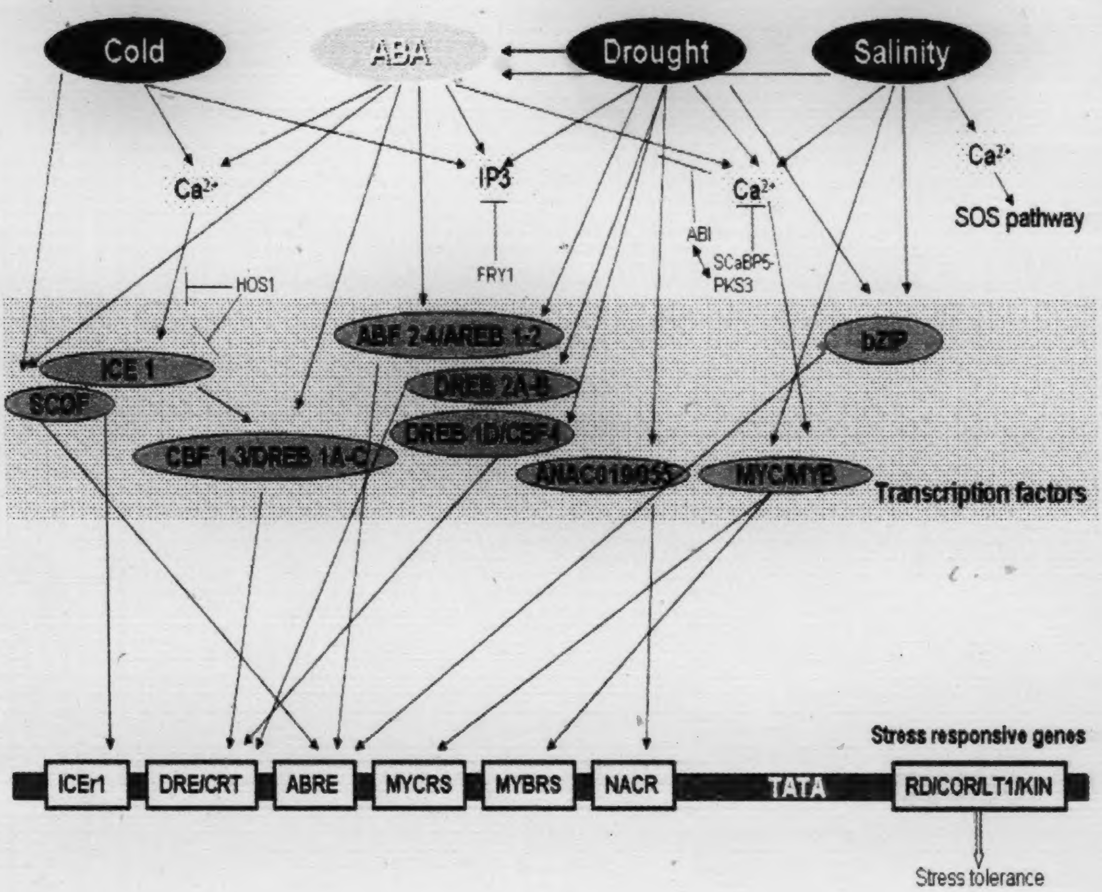

Cold
ABA
Drought
Salinity
Ca2+
IP3
Ca2+
Ca2+
SOS pathway
HOS1
FRY1
ABI
SCaBP5-PKS3
ABF 2-4/AREB 1-2
bZIP
ICE 1
DREB 2A-B
SCOF
DREB 1D/CBF4
CBF 1-3/DREB 1A-C
ANAC019055
MYC/MYB
Transcription factors
Stress responsive genes
ICEr1
DRE/CRT
ABRE
MYCRS
MYBRS
NACR
TATA
RD/COR/LT1/KIN
Stress tolerance

Figure 1-1.

During osmotic stress signaling DREB2A and DREB2B transcription factors activate the DRE *cis*-elements of a stress-responsive genes in ABA-independent pathways (Liu and Zhu, 1998); however, transgenic analysis of *CBF4*, a DREB1 related transcription factor, suggested that regulation of DRE elements can also be mediated by an ABA-dependent pathway (Haake *et al.*, 2002). In *Arabidopsis*, the transcription factors DREB1 (or CBF) and DREB2 bind to the DRE/CRT *cis*-acting elements which further activate the transcription of various stress responsive genes (Liu and Zhu, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Figure 1-1).

Stress responsive genes such as *RD22* contain MYCRS (MYC recognition sequence) and MYBRS (MYB recognition sequence) elements and transcription factors RD22BP1 (a MYC transcription factor), AtMYB2 (a MYB transcription factor) bind to these recognition sequences thus activating their expression (Abe *et al.*, 1997). The SCOF-1 (soybean zinc finger protein) transcription factor which is responsive to ABA and cold, was isolated from Soybean and failed to bind to CRT/DRE or ABRE elements. However, yeast two-hybrid studies revealed interaction of SCOF1 with SGBF1 (Soybean G-box binding bZIP transcription factor). Furthermore, *in vitro* DNA binding activity of SGBF1 to ABRE elements greatly enhanced the levels of SCOF1 (Kim *et al.*, 2001). Therefore, it was suggested that interaction of SCOF1-SGBF1 regulates stress-responsive gene expression via the ABRE in the ABA-dependent pathway (Kim *et al.*, 2001). A zinc finger transcriptional repressor (STZ/ZAT10) may also regulate stress-responsive gene expression in *Arabidopsis*. *LOS2 (low expression of osmotically responsive genes)* encodes an enolase and acts as a repressor of STZ/ZAT10 (a zinc finger transcriptional repressor) (Lee *et al.*, 2002). Transcription factors such as CBF/DREB1 and DREB2 belong to the Apetala 2 (AP2)/ ethylene response element binding protein (EREBP) family, which binds to DRE/CRT *cis*-element and activate transcription of genes *RD29A, RD17, COR 6.6, COR 15a, ERD10, KIN1, KIN2* and others in response to cold and water stress (Liu *et al.*, 1998; Jaglo *et al.*, 2001; Yamaguchi-Shinozaki and Shinozaki, 2006).

1.4.6. Phospholipids signaling during osmotic stress

The plasma membrane plays a crucial role in perception and transmission of external stimuli. Osmotic stress results in changing the phospholipid composition in plants and may serve as a precursor to generate signaling molecules like inositol 1,4,5-triphsophate (IP3), diacylglycerol (DAG) and phosphatidic acid (PA) (Xiong and Zhu, 2002; Mahajan and Tuteja, 2005). One of the most studied phospholipases, phospholipase C (PLC), catalyzes the hydrolysis of phosphatidylinositol 4-5-bisphosphate (PIP2) to generate the secondary messengers IP3 and DAG (Xiong and Zhu, 2002) which may activate protein kinase C and trigger Ca^{2+} release. In *Arabidopsis*, IP3 levels increase under salt stress (Dewald *et al.*, 2001) and osmotic stresses (Takahashi *et al.*, 2001). During salt stress, IP3 increases Ca^{2+} levels, which leads to stomatal closure and water retention in cells (Sanders *et al.*, 1999). Transgenic studies describing the overexpression of inositol polyphosphate 5-phosphate (Ins-5-Pase) have suggested that modification of Ins 5-Pase may regulate IP3 levels and affect stress and ABA signal transduction (Burnette *et al.*, 2001).

Drought or salt stress also increases PI-PLC isoforms in *Arabidopsis* (Hirayama *et al.*, 1995) and potato (Kopka *et al.*, 1998). Phospholipase D (PLD) is involved in membrane lipid hydrolysis, cell signaling and cleaving membrane phospholipids to produce PA and free head groups (Katagiri *et al.*, 2001). Osmotic stress activates phospholipase D (PLD) activity in tomato and alfalfa (Munnik *et al.*, 2000). Overexpression of PLD δ in *Arabidopsis* enhances freezing tolerance (Li *et al.*, 2004).

1.5. Genetic engineering strategies for abiotic stress tolerance in plants

Abiotic stresses such as salinity, drought, low and high-temperatures, as well as oxidative stresses, are often interlinked and they activate similar signalling pathways that may induce similar cellular damages (Knight and Knight, 2001; Zhu *et al.*, 2002; Wang *et al.*, 2003; Vincour and Altman, 2005). From the literature presented earlier it is clear that the mechanism (s) of abiotic stress signalling is very complex and poorly understood. However molecular, physiological, biochemical, genomic, proteomic and transcriptomic approaches have revealed a large number of genes and proteins responding to abiotic stresses. Increased understanding of the regulation of gene expression in response to various abiotic stresses has also revealed the involvement of complex gene networks and cross-talk in mediating abiotic stress responsive gene expression (Shinozaki *et al.*, 2003).

Proteome analysis (using two-dimensional gel electrophoresis and mass spectrometry, Salekdeh *et al.*, 2002; Askari *et al.*, 2006) and transcriptome analysis (using microarrays, Ozturk *et al.*, 2002; Wong *et al.*, 2006) are powerful tools to analyze protein and transcript levels and studies using such techniques have resulted in identification of several genes induced by abiotic stresses. Gene expression studies in various species suggest that plant responses to abiotic stresses may be ubiquitous and mediated through similar mechanisms. The number of genes involved in mediating abiotic stress tolerance can be categorized into the following groups (as described below) and genes from each of these groups have been utilized for making transgenic plants tolerant to abiotic stress conditions (Table 1-1).

Table 1-1. Transgenic strategies for enhancing abiotic stress tolerance

Mechanism		**Gene (Species)**	**Effects on transgenic plants/ comments**	**References**
1.5.1. Ion transporters	**Ion Transport**	*AtNHX1* (*Arabidopsis thaliana*)	Salt stress tolerance	Apse *et al.*, 1999
		AtNHX1 (*B. napus*)	Enhanced salt tolerance; salt accumulates in foliage but not in fruit	Zhang *et al.*, 2001
		AtNHX1 (*Lycopersicon esculentum*)	Enhanced salt tolerance; seed yield and oil quality are not affected by soil salinity	Zhang and Blumwald, 2001
		HAL1 (*Cucurbita melo*)	Yeast *HAL1* gene increases fruit yield and enhances K$^+$/Na$^+$ selectivity under salt stress	Rus *et al.*, 2001
		AVP1 (*A. thaliana*)	Vacuolar H+-pyrophosphatase enhances drought and salt tolerance	Gaxiola *et al.*, 2001
		SOS1 (*A. thaliana*)	*SOS1* gene encoding a plasma membrane Na$^+$/H$^+$ antiporter, improves salt tolerance; less Na$^+$ accumulates in xylem transpirational stream and in shoot	Shi *et al.*, 2003
		NtHAL3 (*N. tabacum*)	*NtHAL3* gene inhibits the activity of protein phosphatases and increases levels of proline and salt tolerance	Yonamine *et al.*, 2004
		AtNHX1 (*Tritivum aestivum*)	Enhances salt tolerance	Xue *et al.*, 2004
		AtNHX1 (*Gossypium hirsutum*)	Enhances salt tolerance	He *et al.*, 2005

		SsVP (*A. thaliana*)	Vacuolar H^+-pyrophosphatase gene, *SsVP*, enhances salt and drought tolerance	Guo *et al.*, 2006
1.5.2. Transcription factors and signalling related genes	**Transcription factors**	*CBF1* (*A.thaliana*)	Induces *COR* genes and enhances freezing tolerance	Jaglo-Ottosen *et al.*, 1998
		DREB1A (*A. thaliana*)	Drought, salt, and freezing tolerance	Kasuga *et al.*, 1999
		HOS2 (*A. thaliana*)	Enhances expression of *RD29A* and other stress genes in *HOS* mutants and enhances freezing tolerance	Lee *et al.*, 1999
		CBF3 (*A. thaliana*)	Increases freezing tolerance of cold-acclimated plants	Gilmour *et al.*, 2000
		CBFs (*Brassica napus*)	Induces expression of *Arabidopsis* CBF-targeted orthologs, increased freezing tolerance in non-acclimated and cold-acclimated plants	Jaglo *et al.*, 2001
		AtGSK1 (*A. thaliana*)	Overexpression of *Arabidopsis* homologue of GSK3/shaggy-like kinase, *AtGSK1*, enhances tolerance to salt stress	Piao *et al.*, 2001
		CBF1 (*L.esculentum*)	Induces catalase; increases chilling tolerance	Hsieh *et al.*, 2002
		CBF4 (*A. thaliana*)	Activates C-repeat/ dehydration responsive elements and freezing and drought tolerance	Haake *et al.*, 2002
		ABF3/ABF4 (*A. thaliana*)	ABA hypersensitivity and other ABA-associated phenotypes and enhances drought tolerance	Kang *et al.*, 2002
		Spl7 (*Oryza sativa*)	Mutant analysis of rice spotted leaf gene, *Spl7* suggestes this is a heat stress transcription factor protein	Yamanouchi *et al.*, 2002
		AtDREB2A (*Nicotiana tabacum*)	EREBP/AP2-type DNA binding protein AtDREB2A increases the tolerance to salt	Shen *et al.*, 2003
		AtMYC2 *AtMYB2* (*A. thaliana*)	ABA-induces gene expression of *rd22* and *AtADH1*; AtMYC2 and AtMYB2 functions as transcriptional activator of ABA induces gene expression under drought stress	Abe *et al.*, 2003
		NPK1 (*Zea mays*)	Constitutive expression of the Nicotiana protein kinase 1 (MAPKKK/*NPK1*) gene enhances freezing tolerance	Shou *et al.*, 2004

		ABF3 (*A. thaliana*)	Enhances freezing, heat and oxidative stress tolerance	Kim *et al.*, 2004
		OSISAP1 (*A. thaliana*)	Overexpression of a zinc-finger protein enhances tolerance to cold, dehydration, and salt stress	Mukhopadhyay *et al.*, 2004
1.5.2. Transcription factors and signalling related genes	**Transcription factors**	*CpMYB10* (*A. thaliana*)	Overexpression of *CpMYB10* cDNA enhances desiccation and salt tolerance	Villalobos *et al.*, 2004
		HOS9 (*A. thaliana*)	*HOS9*, a homeodomain transcription factor gene, enhances cold tolerance through a CBF-independent pathway	Zhu *et al.*, 2004
		CBF3 and *ABF3* (*Oryza sativa*)	Increases tolerance to abiotic stresses	Oh *et al.*, 2005
		AREB1 (*A. thaliana*)	Enhances drought stress tolerance	Fujita *et al.*, 2005
		MBF1c (*A. thaliana*)	Expression of transcriptional co-activator multi protein bridging factor 1c (MBF1c) enhances tolerance of transgenic plants to bacterial infection, heat, and osmotic stress	Suzuki *et al.*, 2005
		CaPF1 (*Pinus virginiana*)	Overexpression of an ERF/AP2 pepper transcription factor (CaPF1) enhances tolerance to heavy metals and heat stress	Tang *et al.*, 2005
	Kinases	*OsCDPK7* (*O. sativa*)	Over-expression of a Ca^{2+}-dependent protein kinase enhances cold, salt and drought tolerance and induces expression of *RAB16*	Saijo *et al.*, 2000
		NPK1 (*N. tabacum*)	Increases tolerance to heat shock, freezing and salt stress	Kovtun *et al.*, 2000
		AtNDPK2 (*A. thaliana*)	Overexpression of *AtNDPK2* (NDP kinase2) induces *AtMPK3* and *6*; enhances multiple stress tolerance	Moon *et al.*, 2003
		OsMAPK5 (*O. sativa*)	Increases tolerance to drought, salt and cold stresses	Xiong and Yang, 2003
		BvCaM (*N. tabacum*)	Overexpression of bovine CaM enhances germination under saline conditions	Olsson *et al.*, 2004
		AtCaMBP25 (*A. thaliana*)	Inhibition of seedling germination under saline and osmotic conditions suggests that *AtCAMBP25* is a negative regulator of osmotic stress response	Perruc *et al.*, 2004
		SRK2C (*A. thaliana*)	Overexpresion of *SRK2C*, a SNF1-related protein kinase 2, enhances drought tolerance	Umezawa *et al.*, 2004

		CNAtr (*O. sativa*)	Expression of a calcineurin gene, a Ca^{2+} and calmodulin dependent serine/threonine phosphatases, enhances salt stress tolerance	Ma *et al.*, 2005
1.5.3. Genes for radical scavenging and detoxification	**Antioxidants**	*Cu/Zn-SOD* (*N. tabacum*)	Overexpression of a pea gene encoding chloroplast-localized *Cu/Zn SOD* increases resistance to oxidative stress	Gupta *et al.*, 1993
		CuZn-SOD (*N. tabacum*)	Overexpression of pea cytosolic *Cu/Zn-SOD* gene enhances resistance to ozone-induced foliar necrosis	Pitcher and Zilinskas, 1996
		Mn-SOD (*Medicago sativa*)	Enhances water deficit stress tolerance	McKersie *et al.*, 1996
		Fe-SOD (*N. tabacum*)	Enhances oxidative stress tolerance	Van Camp *et al.*, 1996
		GST and *GPX* (*N. tabacum*)	Overexpression of a cDNA encoding an enzyme with both glutathione S-transferase (*GST*) and glutathione peroxidase (*GPX*) activity enhances seedling growth during chilling and salt stress	Roxas *et al.*, 1997
		APX (*N. tabacum*)	Increases protection against oxidative stress	Wang *et al.*, 1999^b
		Fe-SOD (*M. sativa*)	Increases winter survival and no oxidative stress tolerance	McKersie *et al.*, 2000
		Aldose-aldehyde reductase (*N. tabacum*)	Protects against lipid peroxidation during drought and oxidative stresses	Oberschall *et al.*, 2000
		ChyB (*A. thaliana*)	Overexpression of *chyB* gene which encodes beta-carotene hydroxylase (an enzyme in zeaxanthin biosynthesis) increased xanthophyll pool increases tolerance to high light and high temperature stress conditions	Davison *et al.*, 2002
		SOD (*Beta vulgaris*)	Increases tolerance to oxidative stress	Tertivanidis *et al.*, 2004
		GPX (*N. tabacum*)	Plants expressing a glutathione peroxidase like protein in the cytosol (TcGPX) or chloroplasts (TpGPX) enhances oxidative stress	Yoshimura *et al.*, 2004

		Mn-SOD (*O. sativa*)	Enhances drought tolerance	Wang *et al.*, 2005
1.5.3. Genes for radical scavenging and detoxification	**Osmolytes**	*mtlD* (*N. tabacum*)	Overexpression of *mtlD* gene which encodes mannitol 1-phosphate dehydrogenase enhances salt tolerance	Tarczynski *et al.*, 1993
		P5CS (*Nicotiana tabacum*)	D1-pyrroline-5-carboxylate synthetase gene overexpression increases proline production and conferrs increased osmo-tolerance	Kishor *et al.*, 1995
		IMT1 (*N. tabacum*)	Transgenics expressing cDNA encoding myo-inositol O-methyltransferase (*IMT1*) accumulates D-ononitol and enhances salt and drought tolerance	Sheveleva *et al.*, 1997
		codA (*A. thaliana*)	Overexpression of *codA* gene for choline oxidase (enzyme that converts choline into glycinebetaine) enhances accumulation of glycinebetaine and tolerance to salt and cold stress	Hayashi *et al.*, 1997
		stpd1 (*N. tabacum*)	Transgenics expressing a cDNA encoding sorbitol-6-phosphate dehydrogenas (*Stpd1*) hyper accumulates sorbitol which leads to osmotic imbalance and necrotic lesions	Sheveleva *et al.*, 1998
		ProDH (*A. thaliana*)	Antisense transgenics with an *AtProDH* cDNA encoding proline dehydrogenase (ProDH) accumulates proline and enhances freezing and salinity tolerance	Nanjo *et al.*, 1999
		CodA (*Brassica juncea*)	Enhances tolerance to salt stress	Prasad *et al.*, 2000
		*Ots*A and *Ots*B (*O. sativa*)	Overexpression of trehalose biosynthetic genes (*ots*A and *ots*B) enhances trehalose accumulation and conferres high tolerance levels to different abiotic stresses	Garg *et al.*, 2002
		CodA (*O. sativa*)	Enhances salinity tolerance	Mohanty *et al.*, 2002
		Glyoxalase I or II (*N. tabacum*)	Enhances salinity tolerance	Singla-Pareek *et al.*, 2003
		mtlD (*Triticum aestivum*)	Expression of the *mtlD* gene for the biosynthesis of mannitol in wheat improves tolerance to water stress and salinity	Abebe *et al.*, 2003

	Osmolytes	*TPSP* (*O. sativa*)	Expression of a bifunctional fusion (*TPSP*) gene for trehalose-6-phosphate (T-6-P) synthase (TPS) and T-6-P phosphatase (TPP) increases trehalose accumulation and abiotic stress tolerance	Jang *et al.*, 2003
		Adc (*O. sativa*)	Expression of Datura *adc* gene modulates polyamine biosynthetic pathway and confers tolerance to drought stress	Capell *et al.*, 2004
		Spermidine synthase (*A. thaliana*)	Enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes such as *DREB* and stress-protective proteins like rd29A	Kasukabe *et al.*, 2004
		Glyoxalase I or II (*N. tabacum*)	Glyoxalase overexpression enhances heavy-metal (zinc) tolerance and restricted methylglyoxal accumulation and less lipid peroxidation in transgenic plants	Singla-Pareek *et al.*, 2006
1.5.4. Stress related proteins	**HSPs and chaperones**	*HSF1* (*A. thaliana*)	Increases heat shock proteins and enhances thermo tolerance	Lee *et al.* 1995
		Hsp17.7 (*Daucus carota*)	Carrot *Hsp17.7* gene constitutively expressing (CaS lines) or expressing a heat inducible antisense RNA (AH lines) demonstrates increased or decreased thermo-tolerance, respectively	Malik *et al.*,1999
		Hsp21 (*A. thaliana*)	Enhances tolerance to oxidative stress	Harndahl *et al.*, 1999
		DnaK1 (*N. tabacum*)	Enhances salt tolerance	Sugino *et al.*, 1999
		AtHSP17.6A (*A. thaliana*)	*At-HSP17.6A*, encoding a small heat-shock proteins (smHSPs) enhances salt and drought tolerance	Sun *et al.*, 2001
		HSP101 (*O. sativa*)	Overexpression of *Athsp101* results in significant improvement of growth performance during heat stress	Katiyar-Agarwal *et al.*, 2003
	LEA	*HVA1* (*O. sativa*)	Enhances tolerance to water deficit and salt stress	Xu *et al.*, 1996
		HVA1 (*Triticum aestivum*)	Improves biomass productivity under water deficit	Sivamani *et al.*, 2000
		WCS19 (*A. thaliana*)	Overexpression of *WCS19* which is related to group 3 LEA proteins	Ndong *et al.*, 2002

			demonstrates increased freezing tolerance	
	LEA	*HVA1* (*O. sativa*)	Enhances dehydration tolerance via cell membrane protection	Chandra Babu *et al.*, 2004
		WCOR410 (Strawberry)	Overexpression of a wheat acidic dehydrin gene (*WCOR410*) enhances freezing tolerance in strawberry cultivar (*Fragaria* X *anassassa* cv. Chambly)	Houde *et al.*, 2004
		DHN (*A. thaliana*)	Dehydrin (*DHN*) overexpression enhances freezing tolerance	Puhakainen *et al.*, 2004
	Other	*PLDδ* (*A. thaliana*)	Phospholipase D (*PLDδ*) enhances freezing tolerance	Li *et al.*, 2004
		PDH45 (*N. tabacum*)	Overexpression of pea DNA helicase 45 exhibits homology with translation initiation factor eIF-4A and enhances salt tolerance	Sanan-Mishra *et al.*, 2005

1.5.1. Pathogenesis-related (PR) 10 proteins and their role *in planta*

In response to various biotic and abiotic stresses plants manifest adaptive responses by producing or accumulating pathogenesis-related (PR) proteins (Jwa *et al.*, 2001). PR proteins are widely present in the plant kingdom in monocots and dicots; they are expressed during development in a tissue-specific manner, and are differentially induced by different stress conditions, suggesting that these proteins have a general role in plant defense systems (Van Loon and Van Strien, 1999). Based on amino acid sequence homology, serological relationship and biological activity, PR proteins are grouped into at least 14 different classes (Van Loon *et al.*, 1994; Van Loon and Van Strien, 1999). Although the biological function of many PR proteins remains unknown, many PR proteins have been recognized as chitinases (PR 3, 4, 8 and 11), β-1, 3-glucanases (PR 2), thaumatin-like proteins (PR 5), proteinase inhibitors (PR 6), endoproteinase (PR 7), peroxidases (PR 9) and defensins (PR 12) in different plant species (Van Loon *et al.*, 1994; Van Loon and Van Strien, 1999).

Most of the PR proteins are secreted, a notable exception to this rule are proteins that belong to the class 10 family of PR proteins (PR 10), also known as intracellular pathogenesis-related proteins (IPR; van Loon *et al.*, 1994), which were first described in cultured parsley cells upon elicitor treatment (Somssich *et al.*, 1988). PR 10 proteins are small (15-18 kDa), acidic pI, cytosolic (van Loon *et al.*, 1994; Markovic-Housley *et al.*, 2003), and resistant to proteases (Warner *et al.*, 1994). Cytokinin-specific binding proteins (CSBP; Fujimoto *et al.*, 1998) and major latex proteins (MLP; Osmark *et al.*, 1998) are also included in the PR 10 class on the basis of their sequence homology. PR 10 proteins have amino acid sequence similarity with a major food allergen of celery and pollen allergens of trees (Warner *et al.*, 1994), and includes proteins such as Bet v 1 and Ara h 8 (Mittag *et al.*, 2004).

The ubiquitous PR 10 proteins are present in a variety of anthophytes including both monocotyledonous and dicotyledonous plants (Biesiadka *et al.*, 2002); they are developmentally regulated in certain tissues and constitutively expressed in different plant organs such as flowers (Breiteneder *et al.*, 1989, Warner *et al.*, 1994, Constable and Brisson, 1995), pollen grains (Apold *et al.*, 1981), fruits (Vanek-Krebitz *et al.*, 1995) and vegetative organs (Crowell *et al.*, 1992; Mylona *et al.*, 1994; Warner *et al.*, 1994; Breiteneder *et al.*, 1995; Liu and Ekramodoullah, 2003). PR 10 proteins are induced by pathogen attack in a wide variety of plants (Fristensky *et al.*, 1988; Somssich *et al.*, 1988; Ekramoddoullah *et al.*, 1998; McGee *et al.*, 2001; Park *et al.*, 2004; Liu *et al.*, 2006), as well as in response to abiotic stresses such as salinity (Moons *et al.*, 1997; Borsics and Lados, 2002; Hashimoto *et al.*, 2004), drought (Hashimoto *et al.*, 2004), heavy metals (Utriainen *et al.*, 1998), ultraviolet radiation (Rakwal *et al.*, 1999), ozone stress (Agarwal *et al.*, 2002) and cold (Ekramoddoullah *et al.*, 1995, 1998; Liu *et al.*, 2003). These studies suggest that PR 10 proteins have a crucial role in plant defense against abiotic and biotic stresses as well as in general growth and development (Crowell *et al.*, 1992; Barratt and Clark, 1993; Sikorski *et al.*, 1999; Osmark *et al.*, 1998; Wu *et al.*, 2003). The constitutive expression of pea *PR 10* in potato confers resistance to potato early dying disease caused by *Verticillium dahliae* (Chang *et al.*, 1993) whereas there is no resistance to blackleg disease in canola (*Leptosphaeria maculans*; Wang *et al.*, 1999^{b}). However, interestingly, no transgenic approach has been utilized to evaluate the utility of any *PR 10* gene in abiotic stress tolerance.

During the last decade many studies have been performed on PR 10 proteins but the exact biological function(s) of these proteins *in planta* remains unclear. The sequence similarities between a ginseng ribonuclease and PR 10 proteins suggested that these proteins possess RNase activity (Moiseyev *et al.*, 1994). PR 10 proteins from *Betula* spp. Bet v 1 (Bufe *et al.*, 1996; Swoboda *et al.*, 1996), white lupin (Bantignies *et al.*, 2000), *Pachyrrhizus erosus* (Wu *et al.*, 2002), cotton (Zhou *et al.*, 2002), hot pepper (Park *et al.*, 2004) and *Solanum* spp. (Liu *et al.*, 2006) possess RNase activity, suggesting that these proteins are involved directly in defense against pathogenic infections or during programmed cell death around infection sites (Liu and Ekramoddoullah, 2004). Such a role has been demonstrated in PR 10 protein from hot pepper which functions as a ribonuclease in an antiviral pathway (Park *et al.*, 2004). RNase activity of pollen grains has been suggested to be involved in recognition and self-incompatibility during fertilization (Huang *et al.*, 1994; Swoboda *et al.*, 1996), and identification of PR 10 protein in the tapetum of lily anthers suggests a potential role in the sporopollenin synthesis pathway (Bolsamo *et al.*, 1995). *PR 10* gene expression is activated by ABA in pea (Iturriaga *et al.*, 1994), by cytokinins in periwinkle callus (Carpin *et al.*, 1998), by jasmonate in rice (Hashimoto *et al.*, 2004), as well by other phytohormones in other plant species. PR 10 proteins also bind many types of molecules such as cytokinins (Fujimoto *et al.*, 1998; Gonneau *et al.*, 2001; Mogensen *et al.*, 2002), brassinosteroids (Markovic-Housley *et al.*, 2003), fatty acids and flavonoids (Mogensen *et al.*, 2002) suggesting that these proteins are involved in general plant growth and development (Liu and Ekramodoullah, 2003; Markovic-Housley *et al.*, 2003). ABA-responsive proteins (e.g. pea ABR17) are homologous to dehydrins and late embryogenesis abundant (LEA) proteins (Skriver and Mundy 1990; Close *et al.*, 1993), which play a protective role against the deleterious effects of dehydration (Pneuli *et al.*, 2002). Therefore, it is possible that ABR17 proteins may have a function similar to dehydrins or LEA.

Many members of the Fabacae family possess PR 10 proteins: drr49 in pea (*Pisum sativum*; Fristensky *et al.*, 1985; 1988); Abscisic acid (ABA) responsive ABA 17 and ABR 18 in pea (Iturriga *et al.*, 1994); PvPR1 and PvPR2 in bean (Walter *et al.*, 1990); SAM22 and H4 in soybean (*Glycine max*; Crowell *et al.*, 1992); MsPR 10 in alfalfa (*Medicago truncatula*; Breda *et al.*, 1996; Gamas *et al.*, 1998); LaPR 10 in white lupin (*Lupinus albus*; Bantignies *et al.*, 2000); LlPR 10 in yellow lupine (*Lupinus luteus*; Sikorski *et al.*, 1995); SPE16 in yam-bean (*Pachyrhizus erosus*; Wu *et al.*, 2002); desert legume (*Retama raetam*; Pneuli *et al.*, 2002) and proteins with allergenic characteristic such as Ara h 8 from peanut (*Arachis hypogaea*; Mittag *et al.*, 2004). PR 10 proteins from white lupin (Bantignies *et al.*, 2000), yellow lupin (Biesiadka *et al.*, 2002) and SPE16 (Wu *et al.*, 2003) also possess RNase activity.

Pea PR 10 proteins are encoded by a multigene family and comprised of five known members such as Drr49a-c (also known as PR 10.1-10.3), and ABA-responsive proteins (ABR) 17 and 18 (also known as PR 10.4 and 10.5) (Tewari *et al.*, 2003). Pea ABR17 and 18 are expressed during seed development and induced by exogenous application of ABA (Iturriaga *et al.*, 1994; Colditz *et al.*, 2004). A relationship between the expression of PR 10 protein and disease resistance has been demonstrated

(Riggleman *et al.*, 1985), but its RNase activity has not been reported. Most PR 10 proteins from legumes such as pea (Mylona *et al.*, 1994), bean (Walter *et al.*, 1996), soybean (Crowell *et al.*, 1992), alfalfa (Breda *et al.*, 1996), yellow lupin (Sikorski *et al.*, 1999) and white lupin (Bantignies *et al.*, 2000) occur in high concentrations in roots. Roots are most exposed to environmental stresses such as drought or salinity, and a higher expression of PR 10 protein in roots may be part of a constitutive defense mechanism (Crowell *et al.*, 1992; Liu *et al.*, 2006). Based on the above mentioned observations we were therefore interested in investigating (1) the levels of PR 10 proteins in pea roots due to salt stress, (2) a possible role of pea PR 10 in plant abiotic stress tolerance, (3) its RNase activity, and (4) the putative role of these proteins.

1.6. Objectives

The broad objective of the research described in this dissertation was to test the following hypotheses:

1. The pea root proteome exhibits significant salinity-induced changes, including the increase in PR 10 levels.
2. Pea PR 10 proteins are important in mediating plant responses to stress, and protect plants from its deleterious effects.
3. Pea PR 10 proteins exhibits RNase activity and this activity mediates abiotic stress response in plants.

We employed a canola (*Brassica napus* L. cv. Westar) line that constitutively expressed a pea *PR 10* gene (*PR 10.1*), an *Arabidopsis thaliana* system that constitutively expressed another pea *PR 10* gene (*PR 10.4/ABR17*), and an *Escherichia coli* expression system to achieve the above objectives and the results are described in subsequent chapters. In the next chapter, I describe the results of a study investigating salinity-induced changes in the pea root proteome which demonstrated that the levels of several members of the pea PR 10 protein family were increased in salinity-stressed roots as previously hypothesized.

1.7. References

Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosakawa, D. and Shinozaki, K. (1997) Role of *Arabidopsis* MYC and MYB homologs in drought- and abscisic acid regulated gene expression. Plant Cell **9:** 1859–1868.

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell **15:** 63–78.

Abebe, T., Guenzi, A.C., Martin, B. and Cushman, J.C. (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol **131:** 1748–1755.

Agrawal, G.K., Rakwal, R., Yonekura, M., Kubo, A. and Saji, H. (2002) Proteome analysis of differentially displayed proteins as a tool for investigating ozone stress in rice (*Oryza sativa* L.) seedlings. Proteomics **2:** 947–959.

Albrecht, V., Ritz, O., Linder, S., Harter, K. and Kudla, J. (2001) The NAF domain deWnes a novel protein–protein interaction module conserved in Ca^{2+}-regulated kinases. EMBO J **20:** 1051–1063.

Amaya, I., Botella, M.A., de la Calle, M., Medina, M.I., Heredia, A., Bressan, R.A., Hasegawa, P.M., Quesada, M.A. and Valpuesta, V. (1999) Improved germination under osmotic stress of tobacco plants overexpressing a cell wall peroxidase. FEBS Lett **457:** 80–84.

Amtmann, A. and Sanders, D. (1999) Mechanisms of Na^+ uptake by plant cells. Adv Bot Res **29:** 75–112.

Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373-399.

Apold, J., Florvaag, E. and Elsayed, S. (1981) Comparative studies on tree-pollen allergens: I. Isolation and partial characterization of a major allergen from birch pollen (*Betula verrucosa*). Int Arch Allergy Appl Immunol **64:** 439–447.

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science **285:** 1256-1258.

Askari, H., Edqvist, J., Hajheidari, M., Kafi, M. and Salekdeh, G.H. (2006) Effects of salinity levels on proteome of *Suaeda aegyptiaca* leaves. Proteomics **6:** 2542-2554.

Bantignies, B., Seguin, J., Muzac, I., Dedaldechamp, F., Gulick, P. and Ibrahim, R. (2000) Direct evidence for ribonucleolytic activity of a PR 10 like protein from white lupin roots. Plant Mol Biol **42:** 871–881.

Barratt, D.H.P. and Clark, J.A. (1993) A stress-induced, developmentally regulated, highly polymorphic protein family in *Pisum sativum*. Planta **191:** 7–17.

Biesiadka, J., Bujacz, G., Sikorski, M.M. and Jaskolski, M. (2002) Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. J Mol Biol **319:** 1223–1234.

Blumwald, E., Aharon, G.S. and Apse, M.P. (2000) Sodium transport in plant cells. Biochim Biophys Acta **1465:** 140–151.

Bohnert, H.J. and Jensen, R.G. (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotech **14:** 89–97.

Bohnert, H.J. and Sheveleva, E. (1998) Plant stress adaptations, making metabolism move. Curr Opin Plant Biol **1:** 267–274.

Bolsamo, R.A., Wang, J.L., Eckard, K.J., Wang, C.S. and Lord, E.M. (1995) Immuogold localization of a developmentally regulated, tapetal-specific, 15 kDa lily anther protein. Protoplasma **189:** 17–25.

Borsics, T. and Lados, M. (2002) Dodder infection induces the expression of a pathogenesis-related gene of the family PR 10 in alfalfa. J Exp Bot **53:** 1831–1832.

Botella, J.R. and Arteca R.N. (1994) Differential expression of two calmodulin genes in response to physical and chemical stimuli. Plant Mol Biol **24:** 757–766.

Bouche, N., Yellin, A., Snedden, W.A. and Fromm, H. (2005) Plant-specific calmodulin-binding proteins. Annu Rev Plant Biol **56:** 435–466.

Boyer, J.S. (1982) Plant productiviy and the environment. Science **218:** 443–448.

Breda, C., Sallaud, C., El-Turk, J., Buffard, D., de Kozak, I., Esnault, R. and Kondorosi, A. (1996) Defense reaction in *Medicago sativa*: a gene encoding a class 10 PR protein is expressed in vascular bundles. Mol Plant Microbe Interact **9:** 713–719.

Breiteneder, H. Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. and Breitenbach, M. (1989) The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. EMBO J **8:** 1935–1938.

Breiteneder, H., Hoffmann-Sommergruber, K., Riordain, G., Susani ,M., Ahorn, H., Ebner, C., Kraft, D. and Scheiner, O. (1995) Molecular characterization of *Api g* 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17 kDa tree pollen allergens. Eur J Biochem **233:** 484–489.

Bufe, A., Spangfort, H., Kahlert, M., Schlaak, M. and Becker, W.M. (1996) The major birch pollen allergen, Bet v 1, shows ribonuclease activity. Planta **199:** 413–415.

Burnette, R.N., Gunesekera, B.M. and Gillaspy, G.E. (2003) An *Arabidopsis* inositol 5-phosphatase gain-of-function alters abscisic acid signaling. Plant Physiol **132:** 1011-1019.

Capell, T., Bassie, L. and Christou, P. (2004) Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Proc Natl Acad Sci USA **101:** 9909–9914.

Carpin, S., Laffer, S., Schoentgen, F., Valenta, R., Chenieux, J.-C., Rideau, M. and Hamdi, S. (1998) Molecular characterization of a cytokinin-inducible periwinkle protein showing sequence homology with pathogenesis-related proteins and the Betv 1 allergen family. Plant Mol Biol **36:** 791–798.

Chandra Babu, R., Zhang, J., Blum, A., Hod, T.H.D., Wue, R. and Nguyen, H.T. (2004) HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. Plant Sci **166:** 855–862.

Chang, M.M., Chiang, C.C., Martin, W.M. and Hadwiger, L.A. (1993) Expression of a pea disease resistance response gene in the potato cultivar Shepody, Amer Potato J **70:** 635–647.

Chartzoulakis, K. and Klapaki, G. (2000) Response of two green house pepper hybrids to NaCl salinity during different growth stages. Sci Hortic **86:** 247–260.

Chen, T.H. and Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol **5:** 250–257.

Cheng, N.H., Pittman, J.K., Zhu, J.K. and Hirschi, K.D. (2004) The protein kinase SOS2 activates the *Arabidopsis* H^+/Ca^+ antiporter CAX1 to integrate Ca^{++} transport and salt tolerance. J Biol Chem **279:** 2922–2926.

Chinnusamy, V., Schumaker, K. and Zhu, J.K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot **55:** 225–236.

Close, T.J., Fenton, R.D., Yang, A., Asghar, R., DeMason, D.A., Crone, D.E., Meyer, N.C. and Moonan, F. (1993) Dehydrin: The protein. In: Plant responses to cellular dehydration during environmental stress (Close, T.J.

and Bray, E.A., eds), pp. 104–118. Rockville, MD: American Society of Plant Physiologists).

Colditz, F., Nyamsuren, O., Niehaus, K., Eubel, H., Braun, H.P. and Krajinski, F. (2004) Proteomic approach: identification of *Medicago truncatula* proteins induced in roots after infection with the pathogenic oomycete *Aphanomyces euteiches*. Plant Mol Biol **55:** 109–120.

Constabel, P. and Brisson, N. (1995) Stigma- and vascular-specific expression of the PR 10a gene of potato: a novel pattern of expression of a pathogenesis-related gene, Mol Plant-Microbe Interact **8:** 104–113.

Crowell, D.N., John, M.E., Russell, D. and Amasino, R.M. (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol Biol **18:** 459–466.

Davenport, R.J. and Tester, M. (2000) A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat. Plant Physiol **122:** 823–834.

Davison, P.A., Hunter, C.N. and Horton, P. (2002) Overexpression of carotene hydroxylase enhances stress tolerance in *Arabidopsis*. Nature **418:** 203–206.

Delauney, A.J. and Verma, D.P.S. (1993) Proline biosynthesis and osmoregulation in plants. Plant J **4**: 215–223.

Delumeau, O., Morere-Le Paven, M.C., Montrichard, F. and Laval-Martin, D.L. (2000) Effects of short-term NaCl stress on calmodulin transcript levels and calmodulin-dependent NAD kinase activity in two species of tomato. Plant Cell Environ **23**: 329–336.

DeWald, D.B., Torabinejad, J., Jones, C.A., Shope, J.C. and Cangelosi, A.R. (2001) Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*. Plant Physiol **126**: 759–769.

Diamant, S., Eliahu, N., Rosenthal, D. and Goloubinoff, P. (2001) Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. J Biol Chem **276:** 39586–39591.

Ekramoddoullah, A.K.M., Davidson, J.J. and Taylor, D. (1998) A protein associated with frost hardiness of western white pine is up-regulated by infection in the white pine blister rust pathosystem. Can J For Res **28:** 412–417.

Ekramoddoullah, A.K.M. and Davidson, J.J. (1995) A method for the determination of conifer foliage protein extracted using sodium dodecyl sulfate and mercaptoethanol. Phytochem Anal **6:** 20–24.

Epstein, E., Norlyn, J.D., Rush, D.W., Kingsbury, R., Kelley, D.B. and Wrana, A.F. (1980) Saline culture of crops: a genetic approach. Science **210:** 399–404.

Essah, P.A., Davenport, R. and Tester, M. (2003) Sodium Influx and Accumulation in *Arabidopsis*. Plant Physiol **133:** 307–318.

FAO. (2005) *Global Network on Integrated Soil Management for Sustainable Use of Salt-affected Soils.* Rome, Italy: FAO Land and Plant Nutrition Management Service.http://www.fao.org/ag/agl/agll/

Finkelstein, R.R., Gampala, S.S. and Rock, C.D. (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell **14:** S15-45.

Flowers, T.J. (1977) The mechanism of salt tolerance in halophytes. Annu Rev Plant Physiol **28:** 89–121.

Flowers, T.J. (2004) Improving crop salt tolerance. J Exp Bot **55:** 307-319.

Flowers, T.J. and Yeo, A.R. (1995) Breeding for salinity resistance in crop plants—where next? Aust J Plant Physiol **22:** 875–884.

Fristensky, B., Riggleman, R.C., Wagoner, W. and Hadwiger, L.A. (1985) Gene expression in susceptible and disease resistant interactions of pea induced with *Fusarium solani* pathogens and chitosan. Physiol Plant Pathol **27:** 15–28.

Fristensky, B., Horovitz, D. and Hadwiger, L.A. (1988) cDNA sequences for pea disease resistance genes. Plant Mol Biol **11:** 713–715.

Fujimoto, Y., Nagata, R., Fukasawa, H., Yano, K., Azuma, M., Iida, A., Sugimoto, S., Shudo, K. and Hashimoto, Y. (1998) Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (*Vigna radiata*). Eur J Biochem **258:** 794–802.

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M.M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2005) AREB1 Is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. Plant Cell **17:** 3470–3488.

Gamas, P., de Billy, F. and Truchet, G. (1998) Symbiosis-specific expression of two *Medicago truncatula* nodulin genes, MtN1 and MtN13, encoding products homologous to plant defense proteins. Mol Plant Microbe Interact **11:** 393–403.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V. and Wu, R.J. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA **99:** 15898–15903.

Gaxiola, R., Li, J., Undurraga, S., Dang, L.M., Allen, G.J., Alper, S.L. and Fink, G.R. (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+ pump. Proc Natl Acad Sci USA **98**: 11444–11449.

Gaxiola, R.A., Rao, R., Sherman, A., Grisa, P., Alper, W.S. and Fink, G.R. (1999) The *Arabidopsis thaliana* proton transporters AtNhx1 and Avp1, can function in cation detoxification in yeast, Proc. Natl. Acad. Sci. USA **96:** 1480–1485.

Geisler, M., Frangne, N., Gomes, E., Martinoia, E. and Palmgren, M.G. (2000) The *ACA4* gene of *Arabidopsis* encodes a vacuolar membrane calcium pump that improves salt tolerance in yeast. Plant Physiol **124:** 1814–1827.

Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D. and Thomashow, M.F. (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol **124:** 1854–1865.

Gonneau, M., Pagant, S., Brun, F. and Laloue, M. (2001) Photoaffinity labelling with the cytokinin agonist azido-CPPU of a 34 kDa peptide of the intracellular pathogenesis-related protein family in the moss *Physcomitrella patens*. Plant Mol Biol **46:** 539–548.

Gosti, F., Beaudoin, N., Serizet, C., Webb, A.A., Vartanian, N. and Giraudat, J. (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell **11:** 1897–1910.

Guo, S., Yin, H., Zhang, X., Zhao, F., Li, P., Chen, S., Zhao, Y. and Zhang, H. (2006) Molecular cloning and characterization of a vacuolar H^+-pyrophosphatase gene, SsVP, from the halophyte *Suaeda salsa* and its overexpression increases salt and drought tolerance of *Arabidopsis*. Plant Mol Biol **60:** 41–50.

Gupta, A.S., Heinen, J.L., Holaday, A.S., Burke, J.J. and Allen, R.D. (1993) Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. Proc Natl Acad Sci USA **90:** 1629–1633.

Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F. and Zhang, J.Z. (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. Plant Physiol **130:** 639–648.

Halfter, U., Ishitani, M. and Zhu, J.K. (2000) The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc Natl Acad Sci USA **97:** 3735–3740.

Hare, P.D., Cress, W.A. and Van Staden, J. (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ **21:** 535–553.

Harndahl, U., Hall, R.B., Osteryoung, K.W., Vierling, E., Bornman, J.F. and Sundby, C. (1999) The chloroplast small heat shock protein undergoes oxidation-dependent conformational changes and may protect plants from oxidative stress. Cell Stress Chaperones **4:** 129–138.

Hasegawa, P.M., Bressan, R.A., Zhu, J.-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51:** 463–499.

Hashimoto, M., Kisseleva, L., Sawa, S., Furukawa, T., Komatsu, S. and Koshiba, T. (2004) A novel rice PR 10 protein, RSOsPR 10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. Plant Cell Physiol **45:** 550–559.

He, C., Yan, J., Shen, G., Fu, L., Holaday, A.S., Auld, D., Blumwald, E. and Zhang, H. (2005) Expression of an *Arabidopsis* vacuolar sodium/proton antiporter gene in cotton improves photosynthetic performance under salt conditions and increases fiber yield in the field. Plant Cell Physiol **46:** 1848–1854.

Hernandez, J.A., Olmos, E., Corpas, F.J., Sevilla, F. and del Rio, L.A. (1995) Salt-induced oxidative stress in chloroplast of pea plants. Plant Sci **105:** 151–167.

Hernandez, J.A., Campillo, A., Jimenez, A., Alarcon, J.J. and Sevilla, F. (1999) Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. New Phytol **141:** 241–251.

Hirayama T, Ohto C, Mizoguchi T and Shinozaki K. (1995) A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **25:** 3903–3907.

Houde, M., Dallaire, S., N'Dong, D. and Sarhan, F. (2004) Overexpression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves. Plant Biotech J **2:** 381–387.

Hsieh, T.H., Lee, J.T., Yang, P.T., Chiu, L.H., Charng, Y.Y., Wang, Y.C. and Chan, M.T. (2002) Heterology expression of the *Arabidopsis* C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. Plant Physiol **129:** 1086–1094.

Hua, W., Liang, S. and Lu, Y. (2003) A tobacco (*Nicotiana tabaccum*) calmodulin-binding protein kinase, NtCBK2, is regulated differentially by calmodulin isoforms. Biochem J **376:** 291–302.

Huang, S., Lee, H.-S., Karunanandaa, B. and Kao, T.H. (1994) Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. Plant Cell **6:** 1021–1028.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T. and Shinozaki, K. (2000) Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. Plant J **24:** 655–665.

Ishitani, M., Liu, J., Halfter, U., Kim, C.S., Shi, W. and Zhu, J.K. (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. Plant Cell **12:** 1667–1677.

Ishitani, M., Xiong, L., Lee, H., Stevenson, B. and Zhu, J.K. (1998) HOS1, a genetic locus involved in cold-responsive gene expression in *Arabidopsis*. Plant Cell **10:** 1151–1161.

Iturriaga, E.A., Leech, M.J., Barratt, D.H.P. and Wang, T.L. (1994) Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. Plant Mol Biol **24:** 235–240.

Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T. and Thomashow, M.F. (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiol **127:** 910–917.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M.F. (1998) *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. Science **280:** 104–106.

Jang, I.C., S.J. Oh, J.S. Seo, W.B. Choi, S.I. Song, C.H. Kim, Y.S. Kim, H.S. Seo, Y.D. Choi, B.H. Nahm and J.K. Kim. (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol **131:** 516–524.

Jwa, N.S., Agrawal, G.K., Rakwal, R., Park, C.H. and Agrawal, V.P. (2001) Molecular cloning and characterization of a novel jasmonate inducible pathogenesis-related class 10 protein gene, JIOsPR 10, from rice (*Oryza sativa* L.) seedling leaves. Biochem Biophys Res Commun **286:** 973–983.

Kang, J.Y., Choi, H.I., Im, M.Y. and Kim, S.Y. (2002) *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343–357.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotech **17:** 287–291.

Kasukabe, Y., He, L., Nada, K., Misawa, S., Ihara, I. and Tachibana, S. (2004) Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. Plant Cell Physiol **45:** 712–722.

Katagiri, F., Thilmony, R. and He, S.Y. (2001) In: Somerville CR, Meyerowitz EM (eds) The *Arabidopsis* book. American Society of Plant Biologists, Rockville. doi/10.1199/tab.0009, http://www.aspb.org/publications/arabidopsis

Katiyar-Agarwal, S., Agarwal, M. and Grover, A. (2003) Heat-tolerant basmati rice engineered by over-expression of hsp101. Plant Mol Biol **51:** 677–686.

Kim, J.-B., Kang, J.-Y. and Kim, S.Y. (2004) Over-expression of a transcription factor regulating ABA-responsive gene expression confers multiple stress tolerance. Plant Biotech J **2:** 459–466.

Kim, J.C., Lee, S.H. and Cheng Y.H. (2001) A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. Plant J **25:** 247–259.

Kishor, K.P.B., Hong, Z., Miao, G.H., Hu, C.A.A. and Verma, D.P.S. (1995) Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increase proline production and confers osmotolerance in transgenic plants. Plant Physiol **108:** 1387–1394.

Knight, H., Trewavas, A.J. and Knight, M.R. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J. **12:** 1067–1078.

Knight, H. (2000) Calcium signalling during abiotic stress in plants. Int Rev Cyt **195:** 269–325.

Knight, H. and Knight, M.R. (2001) Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci **6:** 262-267.

Kohorn, B.D. (2001) WAKs; cell wall associated kinases. Curr Opin Cell Biol. **13:** 529–533.

Kopka, J., Pical, C., Gray, J.E. and Muller-Rober, B. (1998) Molecular and enzymatic characterization of three phosphoinositide-specific phospholipase C isoforms from potato.Plant Physiol **116:** 239-250.

Kovtun, Y., Chiu, W.L., Tena, G. and Sheen, J. (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci U S A **14**: 2940-2945.

Kudla, J., Xu, Q., Harter, K., Gruissem, W. and Luan, S. (1999) Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci USA **96:** 4718–4723.

Kurniadie, D. and Redmann, R.E. (1999) Growth and Cl accumulation in soybean cultivars treated with excess KCl in solution culture. Commun Soil Science Plant Analy **30:** 699–709.

Lee, H., Hübel, A. and Schoffl, F. (1995) Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of heat shock proteins and increased thermotolerance in transgenic *Arabidopsis*. Plant J **8:** 603–612.

Lee, H., Xiong, L., Ishitani, M., Stevenson, B. and Zhu, J.K. (1999) Cold-regulated gene expression and freezing tolerance in an *Arabidopsis thaliana* mutant. Plant J **17:** 301-308.

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B. and Zhu, J.K. (2001) The *Arabidopsis* HOS1 gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. Genes Dev **15:** 912–924.

Lee, H., Guo, Y., Ohta, M., Xiong, L., Stevenson, B. and Zhu, J.K. (2002) LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase. EMBO J **21:** 2692-2702.

Leung J. and Giraudat, J. (1998) Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Biol **49:** 199–222.

Li, W., Li, M., Zhang, W., Welti, R. and Wang, X. (2004) The plasma membrane-bound phospholipase D delta enhances freezing tolerance in *Arabidopsis thaliana*. Nat Biotechnol **22:** 427–433.

Liu, J. and Zhu, J.K. (1998) A calcium sensor homolog required for plant salt tolerance, Science **280:** 1943–1945.

Liu, J., Ishitani, M., Halfter, U., Kim, C.S. and Zhu, J.K. (2000) The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. Proc Natl Acad Sci USA **97:** 3730–3734.

Liu, J.-J., Ekramoddoullah, A.K.M. and Yu, X. (2003) Differential expression of multiple PR 10 proteins in western white pine following wounding, fungal infection and cold-hardening. Physiol Plant **119:** 544–553.

Liu, J.-J. and Ekramoddoullah A.K.M. (2003) Root-specific expression of a western white pine PR 10 gene is mediated by different promoter regions in transgenic tobacco. Plant Mol Biol **52:** 103–120.

Liu, X., Huang, B., Lin, J., Fei, J., Chen, Z., Pang, Y., Sun, X. and Tang, K. (2006) A novel pathogenesis-related protein (SsPR 10) from *Solanum surattense* with ribonucleolytic and antimicrobial activity is stress- and pathogen-inducible. J Plant Physiol **163:** 546–556.

Liu, J.J. and Ekramoddoullah, A.K.M. (2004) Characterization, expression and evolution of two novel subfamilies of *Pinus monticola* (Dougl. ex D. Don) cDNAs encoding pathogenesis-related (PR)-10 proteins. Tree Physiol **24:** 1377–1385.

Lo, S.C., Hipskind, J.D. and Nicholson, R.L. (1999) cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defense-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. Mol Plant Microbe Interact **12:** 479–489.

Longstreth, D.J. and Nobel, P.S. (1979) Salinity effects on leaf anatomy. Plant Physiol **63:** 700–703.

Luan, S. (1998) Protein phosphatases and signaling cascades in higher plants. Trends Plant Sci **3:** 271–275.

Luo, G.Z., Wang, H.W., Huang, J., Tian, A.G., Wang, Y.J., Zhang, J.S. and Chen, S.Y. (2005) A putative plasma membrane cation/proton antiporter from soybean confers salt tolerance in *Arabidopsis*. Plant Mol Biol **59:** 809–820.

Lyon, C. (1941) Responses of two species of tomatoes and the F_1 generation to sodium sulphate in the nutrient medium. Bot Gaz **103:** 107–122.

Ma, X., Qian, Q. and Zhu, D. (2005) Expression of a calcineurin gene improves salt stress tolerance in transgenic rice. Plant Mol Biol **58:** 483–495.

Maeda, T., Wurgler-Murphy, S.M. and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369:** 242–245.

Mahajan, S. and Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys **444:** 139-158.

Malik, M.K., Slovin, J.P., Hwang, C.H. and Zimmerman, J.L. (1999) Modified expression of a carrot small heat shock protein gene, Hsp17.7, results in increased or decreased thermotolerance. Plant J **20:** 89–99.

Markovic-Housely, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. and Breitender, H. (2003) Crystal structure of a hypoallergenic isoforms of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J Mol Biol **325:** 123–133.

McGee, J.D., Hamer, J.E. and Hodges, T.K. (2001) Characterization of a PR-10 pathogenesis- related gene family induced in rice during infection with *Magnaporthe grisea*. Mol Plant-Microbe Interact **14:** 877–886.

McKersie, B.D., Bowley, S.R., Harjanto, E. and Leprince, O. (1996) Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. Plant Physiol **111:** 1177–1181.

McKersie, B.D., Murnaghan, J., Jones, K.S. and Bowley, S.R. (2000) Iron-superoxide dismutase expression in transgenic alfalfa increases winter survival without a detectable increase in photosynthetic oxidative stress tolerance. Plant Physiol **122:** 1427–1438.

Mikolajczyk, M., Olubunmi, S.A., Muszynska, G., Klessig D.F. and Dobrowolska, G. (2000) Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. Plant Cell **12:** 165–178.

Mittag, D., Akkerdaas, J., Ballmer-Weber, B.K., Vogel, L., Wensing, M., Becker, W.M., Koppelman, S.J., Knulst, A.C., Helbling, A., Hefle, S.L., Van Ree, R. and Vieths, S. (2004) Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. J Allergy Clin Immunol **114:** 1410–1417.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. and Shinozaki, K. (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **93:** 765–769.

Mogensen, J.E., Wimmer, R., Larsen, J.N. and Spangfort, M.D. (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J Biol Chem **277:** 684–692.

Mohanty, A., Kathuria, H., Ferjani, A., Sakamoto, A. Mohanty, P. Murata, P. and Tyagi, A.K. (2002) Transgenics of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. Theor Appl Genet **106:** 51–57.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. and Yakovlev, G.I. (1994) High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193:** 470–472.

Monroy, A.F., Sangwan, V. and Dhindsa, R.S. (1998) Low temperature signal transduction during cold acclimation: protein phosphatase 2A as an early target for coldinactivation. Plant J **13:** 653–660.

Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.S., Kim, D.H., Nam, J., Bahk, J., Hong, J.C., Lee, S.Y., Cho, M.J., Lim, C.O. and Yun, D.J. (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proc Natl Acad Sci USA **100:** 358–363.

Moons, A., Prinsen, E., Bauw, G. and Van Montagu, M. (1997) Antagonistic effects of Abscisic acid and Jasmonates on salt-stress-inducible transcripts in rice roots. Plant Cell **9:** 2243–2259.

Morales, M.A., Sancheo-Blanco, M.J., Olmos, E., Torrechlas, A. and Alarcon, J.J. (1998) Changes in the growth, leaf water relations and all ultrastructure in *Argyranthemum coronopifolium* plants under saline conditions J Plant Physiol **153:** 174–180.

Mukhopadhyay, A., Vij, S. and Tyagi, A.K. (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. Proc Natl Acad Sci U S A. **101**: 6309–6314.

Munnik, T., Ligterink, W., Meskiene, I., Calderini, O and Beyerly J. (1999) Distinct osmosensing protein kinase pathways are involved in signaling moderate and severe hyperosmotic stress. Plant J **20**: 381–388.

Munnik, T. and Meijer, H.J. (2001) Osmotic stress activates distinct lipid and MAPK signalling pathways in plants. FEBS Lett **498:** 172–178.

Munnik, T., Meijer, H.J.G., Riet, B. ter, Frank, W., Bartels, D. and Musgrave, A. (2000) Hyperosmotic stress stimulates phospholipae D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. Plant J **22:** 147–154.

Mylona, P., Moerman, M., Yang, W.C., Gloudemans, T., Van de Kerckhove, J., van Kammen, A., Bisseling, T. and Franssen, H.J. (1994) The root epidermis-specific pea gene RH2 is homologous to a pathogenesis-related gene. Plant Mol Biol **26:** 39–50.

Nakagami, H., Pitzschke, A. and Hirt, H. (2005) Emerging MAP kinase pathways in plant stress signalling. Trends Plant Sci **10:** 339–346.

Nakashima, K., Fujita, Y., Katsura, K., Maruyama, K., Narusaka, Y., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2006) Transcriptional regulation of ABI3- and ABA-responsive genes including RD29B and RD29A in seeds, germinating embryos, and seedlings of *Arabidopsis*. Plant Mol Biol **60:** 51-68.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kakubari, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. Plant J **18:** 185–193.

Narusaka, Y., Nakashima, K., Shinwari, Z.K., Sakuma, Y., Furihata, T., Abe, H., Narusaka, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. Plant J **34:** 137–148.

Ndong, C., Danyluk, J., Wilson, K.E., Pocock, T., Huner, N.P.A. and Sarhan, F. (2002) Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. Plant Physiol **129:** 1368–1381.

Niu, X., Zhu, J.K., Narasimham, M.L., Bressan R.A. and Hasegawa, P.M. (1993) Plasma membrane H^+-ATPase gene expression is regulated by NaCl in halophyte (*Atriplex nummularia* L.) cell cultures. Planta **190:** 433–438.

Oberschall, A., Deak, M., Torok, K., Sass, L., Vass, I., Kovacs, I., Feher, A., Dudits, D. and Horvath, G.V. (2000) A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses. Plant J **24:** 437–446.

Oh, S.J., Song, S.I., Kim, Y.S., Jang, H.J., Kim, S.Y., Kim, M., Kim, Y.K., Nahm, B.H. and Kim, J.K. (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol **138:** 341–351.

Ohta, M., Hayashi, Y., Nakashima, A., Hamada, A., Tanaka, A., Nakamura, T. and Hayakawa, T. (2002) Introduction of a Na^+/H^+ antiporter gene from *Atriplex gmelini* confers salt tolerance to rice. FEBS Lett **532:** 279–282.

Olsson, P., Yilmaz, J.L., Sommarin, M., Persson, S. and Bulow, L. (2004) Expression of bovine calmodulin in tobacco plants confers faster germination on saline media. Plant Sci **166:** 1595–1604.

Osmark, P., Boyle, B. and Brisson, N. (1998) Sequential and structural homology between intracellular pathogenesis-related proteins and a group of latex proteins. Plant Mol Biol **38:** 1243–1246.

Ozturk, Z.N., Talame, V., Deyholos, M., Michalowski, C.B., Galbraith, D.W., Gozukirmizi, N., Tuberosa, R. and Bohnert, H.J. (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol **48**: 551-573.

Parida, A.K. and Das, A.B. (2005) Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Saf **60:** 324-349.

Park, C.J., Kim, K.J., Shin, R., Park, J.M., Shin, Y.C. and Paek, K.H. (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J **37:** 186–198.

Patharkar, O.R. and Cushman, J.C. (2000) A stress-induced calcium-dependent protein kinase from Mesembryanthemum crystallinum phosphorylates a two-component pseudo-response regulator. Plant J **24:** 679–691.

Perruc, E., Charpenteau, M., Ramirez, B.C., Jauneau, A. and Galaud, J.P. (2004) A novel calmodulin-binding protein functions as a negative regulator of osmotic stress tolerance in *Arabidopsis thaliana* seedlings. Plant J **38:** 410–420.

Piao, H.L., Lim, J.H., Kim, S.J., Cheong, G.W. and Hwang, I. (2001) Constitutive over-expression of AtGSK1 induces NaCl stress responses in the absence of NaCl stress and results in enhanced NaCl tolerance in *Arabidopsis*. Plant J **27:** 305–314.

Pitcher, L.H. and Zilinskas, B.A. (1996) Overexpression of copper/zinc superoxide dismutase in the cytosol of transgenic tobacco confers partial resistance to ozone-induced foliar necrosis. Plant Physiol **110:** 583–588.

Pnueli, L., Hallak-Herr, E., Rozenberg, M., Cohen, M., Goloubinoff, P., Kaplan, A. and Mittler, R. (2002) Mechanisms of dormancy and drought tolerance in the desert legume *Retama raetam*. Plant J **31**: 319–330.

Poovaiah, B.W., Takezawa, D., An, G. and Han, T.J. (1996) Regulated expression of a calmodulin isoform alters growth and development in potato. J Plant Physiol **149:** 553–558.

Prasad, K.V.S.K., Sharmila, P., Kumar, P.A. and Saradhi, P.P. (2000) Transformation of *Brassica juncea* (L.) Czern with bacterial *codA* gene enhances its tolerance to salt stress. Mol Breed **6:** 489–499.

Puhakainen, T., Hess, M.W., Makela, P., Svensson, J., Heino, P. and Palva, E.T. (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in *Arabidopsis*. Plant Mol Biol **54:** 743–753.

Qiu, Q.S., Guo, Y., Dietrich, M., Schumaker, K.S. and Zhu, J.K. (2002) Regulation of SOS1, a plasma membrane Na/H exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc Natl Acad Sci USA **99:** 8436–8441.

Rakwal, R., Agrawal, G.K. and Yonekura, M. (1999) Separation of proteins from stressed rice (*Oryza sativa* L.) leaf tissues by two-dimensional polyacrylamide gel electrophoresis: induction of pathogenesis-related and cellular protectant proteins by jasmonic acid, UV irradiation and copper chloride. Electrophoresis **20:** 3472–3478.

Riggleman, R.C., Fristensky, B. and Hadwiger, L.A. (1985) The disease resistance response in pea is associated with increased mRNAs. Plant Mol Biol **4:** 81–86.

Roberts, S.K. and Tester, M. (1997) A patch clamp study of Na^+ transport in maize roots. J Exp Bot **48:** 431–440.

Roxas, V.P., Smith, R.K. Jr., Allen, E.R. and Allen, R.D. (1997) Overexpression of glutathione *S*-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. Nat Biotech **15:** 988–991.

Rus, A.M., Estaño, M.T., Gisbert, C., Garcia-Sogo, B., Serrano, R., Caro, M., Moreno, V. and Bolarin, M.C. (2001) Expressing the yeast HAL1 gene in tomato increases fruit yield and enhances K^+/Na^+ selectivity under salt stress. Plant Cell Environ **24:** 875–880.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J **23:** 319–327.

Sakamoto, A., Ali, A. and Murata N. (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant Mol Biol **38:** 1011–1019.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. and Bennett, J. (2002) Proteomic analysis of rice leaves during drought stress and recovery. Proteomics **2:** 1131–1145.

Sanan-Mishra, N., Pham, X.H. Sopory, S. K. and Tuteja, N. (2005) Pea DNA helicase 45 overexpression in tobacco confers high salinity tolerance without affecting yield. Proc Natl Acad Sci U S A **102:** 509–514.

Sanders, D., Brownlee, C. and Harper, J.F. (1999) Communicating with calcium. Plant Cell **11:** 691–706.

Schubert, S. and Lauchli, A. (1990) Salt exclusion mechanisms at the root surface of two maize cultivars. Plant Soil **123:** 205–209.

Shen, Y.G., Yan, D.Q., Zhang, W.K., Du, B.X., Zhang, J.S., Liu, Q. and Chen, S.Y. (2003) Novel halophyte EREBP/AP2-type DNA binding protein improves salt tolerance in transgenic tobacco. Acta Bot Sin **45:** 82–87.

Sheen, J. (1996) Ca^{2+}-dependent protein kinases and stress signal transduction in plants. Science **274:** 1900–1902.

Sheen, J. (1998) Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. Proc Natl Acad Sci USA **95:** 975–980.

Sheveleva, E., Chmara, W., Bohnert, H.J. and Jensen, R.G. (1997) Increased salt and drought tolerance by d-ononitol production in transgenic *Nicotiana tabacum* L. Plant Physiol **115:** 1211–1219.

Sheveleva, E.V., Marquez, S., Chmara, W., Zegeer, A., Jensen, R.G. and Bohnert, H.J. (1998) Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco. Plant Physiol **117:** 831–839.

Shi H., Ishitani, M., Kim C. and Zhu J.-K. (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na$^+$/H$^+$ antiporter. Proc Natl Acad Sci USA **97:** 6896–6901.

Shi, H., Lee, B.H., Wu, S.J. and Zhu, J.K. (2003) Overexpression of a plasma membrane Na$^+$/H$^+$ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nature Biotech **21:** 81–85.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol **3:** 217–223.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol **6:** 410–417.

Shou, H., Bordallo, P., Fan, J.B., Yeakley, J.M., Bibikova, M., Sheen, J. and Wang, K. (2004) Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc Natl Acad Sci USA **101:** 3298–3303.

Sikorski, M.M., Biesiadka, J., Kasperska, A.E., Kopcinska, J., Lotocka, B., Golinowski, W. and Legocki, A.B. (1999) Expression of genes encoding PR 10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. Plant Sci **149:** 125–137.

Singla-Pareek, S.L., Reddy, M.K. and Sopory, S.K. (2003) Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. Proc Natl Acad Sci USA **100:** 14672–14677.

Singla-Pareek, S.L., Yadav, S.K., Pareek, A., Reddy, M.K. and Sopory, S.K. (2006) Transgenic tobacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. Plant Physiol. **140:** 613–623.

Sivamani, E., Bahieldin, A., Wraithc, J.M., Al-Niemia, T., Dyera, W.E., Hod, T.H.D. and Qu, R. (2000) Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. Plant Sci **155:** 1–9.

Skriver, K. and Mundy, J. (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512.

Somssich, I.E., Schmelzer, E., Kawalleck, P. and Hahlbrock, K. (1988) Gene structure and in situ transcript localization of pathogenesis-related protein 1 in parsley. Mol Gen Genet **213:** 93–98.

Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA **94:** 1035-1040.

Stone, J.M. and Walker, J.C. (1995) Plant protein kinase families and signal transduction. Plant Physiol **108:** 451–457.

Sugino, M., Hibino, T., Tanaka, Y., Nii, N. and Takabe, T. (1999) Overexpression of DnaK from a halotolerant cyanobacterium *Aphanothece halophytice* acquires resistance to salt stress in transgenic tobacco plants. Plant Sci **146:** 81–88.

Sun, W., Bernard, C., van de Cotte, B., Van Montagu, M. and Verbruggen, N. (2001) At-HSP17.6A, encoding a small heat-shock protein in *Arabidopsis*, can enhance osmotolerance upon overexpression. Plant J **27:** 407–415.

Sussman, M. R. (1994) Molecular analysis of proteins in the plant plasma membrane. Annu Rev Plant Physiol Plant Mol Biol **45:** 211-234.

Suzuki, I., Los, D.A., Kanesaki, Y., Mikami, K. and Murata, N. (2000) The pathway for perception and transduction of low-temperature signals in *Synechocystis*. EMBO J **19:** 1327–1334.

Suzuki, N., Rizhsky, L., Liang, H., Shuman, J., Shulaev, V. and Mittler, R. (2005) Enhanced tolerance to environmental stress in transgenic plants expressing the transcriptional coactivator multiprotein bridging factor 1c. Plant Physiol **139:** 1313–1322.

Swoboda, I., Hoffmann-Sommergruber, K., Riordain, G. O, Scheiner, O., Heberle-Bors, E. and Vicente, O. (1996) Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. Physiol Plant **96:** 433–438.

Takahashi, S., Katagiri, T., Hirayama, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2001) Hyperosmotic stress induced a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in *Arabidopsis* cell culture. Plant Cell Physiol **42:** 214–222.

Tang, W., Charles, T.M. and Newton, R.J. (2005) Overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus Virginiana*

Mill.) confers multiple stress tolerance and enhances organ growth. Plant Mol Biol **59:** 603–617.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science **259:** 508–510.

Taylor, R.J. and Hoxley, G. (2003) Dryland salinity in Western Australia: managing a changing water cycle. Water Sci Technol **47:** 201–207.

Tena, G., Asai, T., Chiu, W.L. and Sheen, J. (2001) Plant mitogen-activated protein kinase signaling cascades. Curr Opin Plant Biol **4:** 392–400.

Tertivanidis, K., Goudoula, C., Vasilikiotis, C., Hassiotou, E., Perl-Treves, R. and Tsaftaris, A. (2004) Superoxide dismutase transgenes in sugarbeets confer resistance to the fungus C. beticola and oxidative agents. Transgenic Res **13:** 225–233.

Tester, M. and Davenport, R. (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann Bot (Lond) **91:** 503–527.

Tewari, S., Brown, S.M., Kenyon, P., Balcerzak, M. and Fristensky, B. (2003) Plant defense multigene families. II Evolution of coding sequence and differential expression of PR 10 genes in *Pisum.* http://arXiv.org/q -bio.PE/0310038

Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanism. Annu Rev Plant Physiol Plant Mol Biol **50:** 571–599.

Tyerman, S.D., Skerrett, M., Garrill, A., Findlay, G.P. and Leigh, R.A. (1997) Pathways for the permeation of Na^+ and Cl^- into protoplasts derived from cortex of wheat roots. J Exp Bot **48:** 459–480.

Ulm, R., Ichimura, K., Mizoguchi, T., Peck, S.C., Zhu, T., Wang, X., Shinozaki, K. and Paszkowski, J. (2002) Distinct regulation of salinity and genotoxic stress responses by *Arabidopsis* MAP kinase phosphatase 1. EMBO Journal **21:** 6483–6493.

Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in *Arabidopsis thaliana.* Proc Natl Acad Sci USA **101:** 17306–17311.

Uozumi, N., Kim, E.J., Rubio, F., Yamaguchi, T. and Muto, S. (2000) The *Arabidopsis* HKT1 gene homolog mediates inward Na currents in *Xenopus laevis* oocytes and Na uptake in *Saccharomyces cerevisiae*. Plant Physiol **122:** 1249–1260.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N. and Shinozaki, K. (1994) Two genes that encode $Ca(^{2+})$-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana.* Mol Gen Genet **244:** 331–340.

Urao, T., Yamaguchi-Shinozaki. K. and Shinozaki, K. (2000) Two-component systems in plant signal transduction. Trends Plant Sci **5:** 67–74.

Utriainen, M., Kokko, H., Auriola, S., Sarrazin, O. and Karenlampi, S. (1998) PR 10 protein is induced by copper stress in roots and leaves of a Cu/Zn tolerant clone of birch, *Betula pendula*. Plant Cell Environ **21:** 821–828.

Van Camp, W., Capiau, K., Van Montagu, M., Inze, D. and Slooten, L. (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. Plant Physiol **112:** 1703–1714.

Van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994) Recommendation for naming plant pathogenesis-related proteins. Plant Mol Biol Rep **12:** 245–264.

Van Loon, L.C. and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR1 type proteins. Physiol Mol Plant Pathol **55:** 85–97.

Vanek-Krebitz, M., Hoffmann-Sommergruber, K., Laimer da Camara Machado, M., Susani, M., Ebner, C., Kraft, D., Scheiner, O. and Breiteneder, H. (1995) Cloning and sequencing of Mal d I, the major allergen from apple (*Malus domestica*), and its immunological relationship to Betv 1, the major birch pollen allergen. Biochem Biophys Res Commun **214:** 538–551.

Villalobos, M.A., Bartels, D. and Iturriaga, G. (2004) Stress tolerance and glucose insensitive phenotypes in *Arabidopsis* overexpressing the CpMYB10 transcription factor gene. Plant Physiol **135:** 309–324.

Vinocur, B. and Altman, A. (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol **16:** 123–132.

Walter, M.H., Liu, J.W., Grand, C., Lamb, C.J. and Hess, D. (1990) Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of proteins including pollen allergens. Mol Gen Genet **222:** 353–360.

Walter, M.H., Liu, J.W., Wunn, J. and Hess, D. (1996) Bean ribonuclease-like pathogenesis-related protein genes (Ypr 10) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. Eur J Biochem **239:** 281–293.

Wang, H., Qi, Q., Schorr, P., Cutler, A.J., Crosby, W.L. and Fowke, L.C. (1998) ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. Plant J **15:** 501–510.

Wang, J., Zhang H. and Allen R.D. (1999[a]) Overexpression of an *Arabidopsis* peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress. Plant Cell Physiol **40:** 725–732.

Wang, Y., Nowak, G., Culley, D., Hadwiger, L.A. and Fristensky, B. (1999[b]) Constitutive expression of pea defense gene DRR206 confers resistance to Blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). Mol Plant-Microbe Interact **12:** 410–418.

Wang, W., Vinocur, B. and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218:** 1–14.

Wang, F.Z., Wang, Q.B., Kwon, S.Y., Kwak, S.S. and Su, W.A. (2005) Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. J Plant Physiol **162:** 465–472.

Warner, S.A.J., Gill, A. and Draper, J. (1994) The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. Plant J **6:** 31–43.

Wegner, L.H. and de Boer, A.H. (1997) Properties of two outward-rectifying channels in root xylem parenchyma cells suggest a role in K+ homeostasis and long-distance signaling. Plant Physiol **115:** 1707–1719.

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inze, D. and Van Camp, W. (1997) Catalase is a sink for H2O2 and is indispensable for stress defense in C3 plants. EMBO J **16:** 4806–4816.

Wong, C.E., Li, Y., Labbe, A., Guevara, D., Nuin, P., Whitty, B., Diaz, C., Golding, G.B., Gray, G.R., Weretilnyk, E.A., Griffith, M. and Moffatt, B.A. (2006) Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in *Thellungiella*, a close relative of *Arabidopsis*. Plant Physiol **140:** 1437-1450.

Wu, F., Li, Y.K., Chang, S., Zhou, Z., Wang, F., Song, X., Lin, Y. and Gong, W. (2002) Purification, characterization and preliminary crystallographic studies of a PR 10 protein from *Pachyrrhizus erosus* seeds. Acta Crystal D **58:** 2165–2167.

Wu, F., Yan, M., Li, Y., Chang, S., Song, X., Zhou, Z. and Gong, W. (2003) cDNA cloning, expression, and mutagenesis of a PR-10 protein SPE-16 from the seeds of *Pachyrrhizus erosus*. Biochem Biophys Res Commun **312:** 761–766.

Xiong, L., Ishitani, M., Lee, H. and Zhu, J-K. (2001) The *Arabidopsis* LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. Plant Cell **13:** 2063–2083.

Xiong, L., Schumaker, K.S. and Zhu, J.-K. (2002) Cell signalling for cold, drought, and salt stresses. Plant Cell **14:** S165–S183.

Xiong, L. and Yang, Y. (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell **15:** 745–759.

Xiong, L. and Zhu, J.-K. (2001) Plant abiotic stress signal transduction: molecular and genetic perspectives. Physiol Plant **112:** 152–166.

Xiong, L. and Zhu, J.-K. (2002) Molecular and genetic aspect of plant responses to osmotic stresses. Plant Cell Environ **25:** 131–139.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D. and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol **110:** 249–257.

Xu, G., Magen, H., Tarchitzky, J. and Kafkafi, U. (2000) Advances in chloride nutrition of plants. Adv Agron **68:** 97-150.

Xue, Z.Y., Zhia, D.Y., Xue, G.-P., Zhangc, H., Zhaoc, Y.-X. and Xia, G.-M. (2004) Enhanced salt tolerance of transgenic wheat (*Tritivum aestivum* L.) expressing a vacuolar Na^+/H^+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na^+. Plant Sci **167:** 849–859.

Yamanouchi, U., Yano, M., Lin, H., Ashikari, M. and Yamada, K. (2002) A rice spotted leaf gene, Spl7, encodes a heat stress transcription factor protein. Proc Natl Acad Sci USA **99:** 7530–7535.

Yamaguchi, T. and Blumwald, E. (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci **10:** 615–620.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251–264.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol **57:** 781-803.

Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus and G.N. Somero (1982) Living with water stress: evolution of osmolyte systems. Science **217:** 1214-1222.

Yeo, A.R., Flowers, S.A., Rao, G., Welfare, K., Senanayake, N. and Flowers, T.J. (1999) Silicon reduces sodium uptake in rice (*Oryza sativa L.*) in saline conditions and this is accounted for by a reduction in the transpirational bypass flow. Plant Cell Environ **22:** 559–565.

Yonamine, I., Yoshida, K., Kido, K., Nakagawa, A., Nakayama, H. and Shinmyo, A. (2004) Overexpression of NtHAL3 genes confers increased levels of proline biosynthesis and the enhancement of salt tolerance in cultured tobacco cells. J Exp Bot **55:** 387–395.

Yoshimura, K., Miyao, K., Gaber, A., Takeda, T., Kanaboshi, H., Miyasaka, H. and Shigeoka, S. (2004) Enhancement of stress tolerance in transgenic tobacco plants overexpressing *Chlamydomonas* glutathione peroxidase in chloroplasts or cytosol. Plant J **37:** 21–33.

Zhang, H.-X. and Bumwald, E. (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nature Biotech **19:** 765–768.

Zhang, H.X., Hodson, J.N., Williams, J.P. and Blumwald, E. (2001) Engineering salt-tolerant *Brassica plants*: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc Natl Acad Sci USA **98:** 12832–12836.

Zhou, X.J., Lu, S., Xu, Y.H., Wang, J.W. and Chen, X.Y. (2002) A cotton cDNA (GaPR-10) encoding a pathogenesis-related 10 protein with *in vitro* ribonuclease activity. Plant Sci **162:** 629–636.

Zhu, J.K., Liu, J. and Xiong, L. (1998) Genetic analysis of salt tolerance in *Arabidopsis*. Evidence for a critical role of potassium nutrition. Plant Cell **10:** 1181–1191.

Zhu, J.-K. (2001^{a}) Plant salt tolerance. Trends Plant Sci **6:** 66–71.

Zhu, J.-K. (2001^b) Cell signaling under salt, water and cold stresses. Curr Opin Biotechnol **9:** 214–219.

Zhu, J.-K. (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Physiol Plant Mol Biol **53:** 247–273.

Zhu, J.-K. (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol **6:** 441–445.

Zhu, J., Shi, H., Lee, B.H., Damsz, B., Cheng, S., Stirm, V., Zhu, J.K., Hasegawa, P.M. and Bressan, R.A. (2004) An *Arabidopsis* homeodomain transcription factor gene, HOS9, mediates cold tolerance through a CBF-independent pathway. Proc Natl Acad Sci USA **101:** 9873–9878.

2. Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity

2.1. Introduction

Salinity is a major abiotic stress in plant agriculture worldwide, resulting in a loss of $US 12 billion annually which is expected to rise as soils are further affected (Pitman and Lauchli, 2002). The United Nations Environment Program (UNEP) estimated that approximately 20% of agricultural land and 50% of cropland in the world are under salinity stress (Flowers and Yeo, 1995). Salinity imposes a type of water-deficit stress that invokes responses in plants that are similar to those elicited by drought stress (Zhu, 2002). Salt stress arises from excessive uptake of salt by plants and is the unavoidable consequence of high ion concentrations in the medium leading to water-deficit stress as a result of the low external water potential and excessive accumulation of ions inside the plant which results in ion toxicity (Greenway and Munns, 1980). Effects of salinity stress include membrane disorganization, generation of toxic metabolites, inhibition of photosynthesis, generation of reactive oxygen species and attenuated nutrient acquisition followed, eventually, by cell and whole plant death (Hasegawa *et al.*, 2000).

A version of this chapter has been published: Kav N.N.V., ***Srivastava S.,*** *Goonewardene L. and Blade S. (2004) Proteome-level changes in the roots of Pisum sativum L. in response to salinity,* ***Annals of Applied Biology****, 145, 217-230.*

Responses of plants to salinity stress and the development of salt tolerance are extremely complex and various mechanisms appear to be involved (Majoul *et al.*, 2000). Generation of salt-tolerant crop plants has been the quest of plant breeders who have used classical approaches that have not proven to be successful. Molecular biological techniques have accelerated the pace of investigation into the identification of gene(s) involved in salt stress response pathways and the validation of some genes as potential targets for improvement of salinity stress tolerance in plants. An example of a signal transduction pathway that has been well characterized in response to salinity stress involves the salt overly sensitive (*SOS*) genes and a calcium-responsive SOS-3/SOS-2 protein kinase complex, which controls the expression and activity of SOS1 ion transporter (Zhu, 2002). Overexpression of the Na^+ efflux carrier in *Arabidopsis* confers increased salinity tolerance, thereby providing evidence for its vital role (Shi and Zhu, 2002). Other salinity and drought stress induced changes include phospholipid hydrolysis; changes in *LEA*/dehydrin-type gene expression, molecular chaperones and proteinases; activation of enzymes involved in the generation and removal of reactive oxygen species and detoxification proteins. However, our understanding of the various pathways involved in plant responses to salinity stress, as well as the interaction and regulation of these pathways, are far from complete (Xiong and Zhu, 2002; Zhu, 2002).

In the post-genomic era, proteomics-based techniques offer powerful approaches for the identification of proteins associated with a particular environmental and/or developmental signal (Gygi and Aebersold, 2000). Protein amounts are not always correlated to mRNA levels (Gygi *et al.*, 1999), probably due to large differences in protein turnover and post-translational modifications and, therefore, only the study of proteins themselves can provide information on their real amount and activity (Zivy and de Vienne, 2000). Plant responses to salinity and other water-deficit stresses such as drought have been investigated using proteomics/genomics-based approaches (Ouerghi *et al.*, 2000; Salekdeh *et al.*, 2002; Wang *et al.*, 2003) but have been limited to non-leguminous plant species. In legumes, the effects of salinity have only been investigated with respect to understanding the role of specific proteins in ameliorating the stress, including the role of antioxidant systems in peas (Hernandez and Almansa, 2002). In order to fully understand the mechanisms involved in mediating the responses of legumes (and perhaps plants in general) to salinity and other water-deficit stresses, we have initiated a research program aimed at identifying and characterizing proteins whose levels are altered by salinity stress in pea, an economically important crop used as human food and animal feed throughout the world. In this paper, we present the identities of 35 root proteins identified by Mass Spectrometry (MS) that were affected by the imposed salinity stress and discuss the role of some of these proteins in mediating responses of pea to salinity stress and their potential utility in the genetic engineering of stress tolerance.

2.2. Materials and Methods

2.2.1. Plant material and imposition of stress

Pea (*Pisum sativum* L. cv. Cutlass) seeds were obtained from Alberta Agriculture, Food and Rural Development (AAFRD) and planted in the greenhouse for long-term experiments. Seeds were sown individually in plastic pots with a diameter of 15.5 cm, containing Metro Mix® 290 (Grace Horticultural products, Ajax, Ontario) in the greenhouse (22°C day/18°C night, 16 h photoperiod) supplemented with Sylvania 400 watt high pressure sodium (HPS) light 940 µmol m^{-2} s^{-1}. The experiment was designed in a randomized complete block design (RCBD) incorporating seven levels of salinity (0, 25, 50, 75, 100, 125 and 150 mM NaCl) over three biological replicates. Each pot with one seed and five pots for each treatment in replicate were used in the whole experiment. Plants were fertilized for the first two weeks with a complete fertilizer (Peters™20-20-20, Plant Products, Bramalea, Ontario) containing micronutrients. After two weeks of normal growth, the plants were watered with 200 ml of deionised water (control) or 25, 50, 75, 100, 125 and 150 mM NaCl solutions on alternate days for six weeks. Morphological appearance of the plants was recorded daily and physical parameters (root length and mass) were measured at regular intervals (2, 4, 6 and 8 weeks) after the commencement of the salt-water regime. The entire experiment was repeated at least three times and the data were statistically analyzed.

In the case of the shorter-term (Petri dish) experiments, pea seeds were surface sterilized for 1 min in 70% ethanol, 5 min in 5 % (v/v) sodium hypochlorite in water and rinsed in sterile deionised water 3-4 times. The seeds (5 per plate) were placed on a sterile filter paper in a Petri dish containing 5 ml of deionised water (control) or 25, 50, 75, 100, 125 and 150 mM NaCl solutions. The plates were sealed with laboratory film and incubated in the dark at room temperature (20±2°C) for 7 days after which, the length of the roots from the tip of the radicle to the cotyledons was measured. Each experiment consisted of three replicates per treatment concentration. The entire experiment was repeated at least three times and the data were statistically analyzed.

2.2.2. Relative water content measurements

Relative water content (RWC) of roots excised from pea seedlings germinated in the presence or absence of NaCl was determined as described by Jin *et al.* (2000). Briefly, fresh weights of excised roots (W_f) were measured after which the roots were allowed to attain turgor by floating on water for 24 h. Fully turgid roots were then weighed (W_t) before they were dried in an oven at 65ºC for 24 h to obtain the dry weights (W_d). RWC was expressed as the water content in the original sample as a percentage of the water in the fully hydrated tissue and was calculated using the formula (Jin *et al.*, 2000):

$RWC = 100 \times [(W_f - W_d)/ (W_t - W_d)]$.

2.2.3. Statistical analysis

Statistical analyses were performed by analysis of variance (ANOVA) procedure using the general linear model (GLM) of SAS (Anon., 1989). Root length, mass or relative water content was analyzed as a one-way treatment structure where the salt concentrations were treatments. Linear trends for treatments were analyzed using orthogonal contrasts. The differences between least square means for fixed effects were established using the standard error of a difference based on a *t*-test when *P*<0.05.

2.2.4. Protein extraction

Protein extracts for two-dimensional electrophoresis were prepared according to the method of Damerval *et al.* (1986), with modifications. Pooled root tissues from three independent experiments were homogenized to a fine powder in liquid nitrogen and 0.2 g of the homogenized tissue material was extracted with 10% w/v TCA in acetone containing 0.07% DTT and the extract was incubated at -17°C for 1 h. The samples were then centrifuged at 13,000 g for 15 min and the pellet was resuspended in ice-cold acetone containing 0.07% DTT and incubated at -17°C for an additional hour. The samples were once again centrifuged as described above and the wash step was repeated two more times. The washed pellet was dried at room temperature for 30 min and 500 µL of the sequential protein extraction reagent 3 (Bio-Rad, Mississauga, Canada) containing 5 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v SB 3-10, 40 mM Tris, 0.2% Bio-lyte 3-10 and 5 µl of 200 mM tributylphosphine (TBP) was added, vortexed vigorously and incubated overnight at 4°C. Samples were subsequently centrifuged at 4°C for 15 min at 13,000 g and the supernatant containing the proteins to be analyzed was transferred in fresh tubes. The concentration of protein in the samples was determined using a modified Bradford assay (Bio-Rad) using BSA as a standard and were stored at -17°C until further analysis.

2.2.5. Two-dimensional electrophoresis

Pea root protein extracts were analyzed by high resolution 2-D PAGE, with isoelectric focusing (IEF) using immobilized pH gradient (IPG) strips in the pH 4-7 range to separate proteins in the first- and SDS-PAGE in the second-dimensions. IEF was carried out using a Bio-Rad IEF system. IPG strips (7cm, 4-7 L, Bio-Rad) were rehydrated overnight at room temperature with a total 125 μL solution containing the extracted protein (100 μg total protein for shorter-term stress and 40 μg total protein for longer-term stress) in the rehydration buffer (Bio-Rad) in a reswelling tray. Rehydrated strips were positioned in the focusing tray and covered with mineral oil and focused in the IEF unit under maximum field strength of ~600 V cm^{-1} and a 50 μA limit/IPG strip. Initially, a low voltage 250 V cm^{-1} for 15 min was applied to remove salt ions and charged contaminants, followed by a linear voltage ramping step to reach 4000 V in 2 h after which focusing took place at constant 4000 V for 20,000 Vhours without exceeding the 50 μA/strip limit. At the end of focusing, a 500 V hold step that was maintained in order to prevent diffusion of proteins and to minimize over-focused artifacts until the run was stopped manually.

The strips were removed from the IEF unit and equilibrated in 10 ml of 50 mM Tris-HCl, pH 8.8, containing 6 M urea, 20% glycerol, 2% w/v SDS and 2% w/v DTT for 10 min at room temperature followed by another 10 min in 10 ml of 50 mM Tris-HCl buffer, pH 8.8, containing 6 M urea, 20% glycerol, 2% w/v SDS and 2.5% w/v iodoacetamide to reduce and alkylate the proteins. Separation in the second dimension was performed by SDS-PAGE using a vertical slab of 13% acrylamide using a Mini

PROTEAN 3 system (Bio-Rad) at constant voltage (160 V) until the dye front reached the bottom of the gel. Protein spots were visualised by staining with Coomassie blue R-250 and subsequent silver staining by first placing them in a solution of Coomassie blue R-250 (0.05% in 40% methanol and 10% acetic acid) for 1 h and destained overnight with 40% methanol and 10% acetic acid and then stained using the Silver Stain Plus kit (Bio-Rad) as per manufacturer's instructions.

2.2.6. Image analysis

2-D gels stained with Coomassie blue followed by silver stain were scanned using a Bio-Rad GS-800 calibrated densitometer. Analysis of the scanned images was performed using the Bio-Rad PDQuest software using the automated detection and matching and spot identification wizard. After automated spot identification, manual validation and addition/removal of spots was performed as required to include spots that were missed by the automated identification or to eliminate artifacts that were mistaken for spots. Each area of the gels from different treatments (Control, 75 and 150 mM NaCl) was systematically analyzed and compared between each other and spots that were not matched during the automated matching process were matched manually or assigned as unique spots. Using default characteristics of PDQuest software (as described by manufacturer) those spots which showed reproducible changes in density (up- or down-regulation) as a result of the salinity treatment, were selected for ESI-Q-TOF MS/MS analysis.

2.2.7. ESI-Q-TOF MS/MS analysis

ESI-Q-TOF MS/MS analysis of the excised protein sample was performed at the Institute for Biomolecular Design (IBD), University of Alberta, Canada. Briefly, spots of interest were excised from the gels using a sterile scalpel and placed in numbered wells of a microtiter plate and submerged with 40 μL of sterile water. Processing of the gel pieces was performed in a fully automated fashion on a Mass Prep Station (Micromass, UK). The gel pieces were de-stained, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with 6 ng/μL trypsin (Promega Sequencing Grade Modified) in 50 mM ammonium bicarbonate (25 μL), for 5 h at 37°C. After enzymatic digestion, the tryptic peptides were extracted and then subjected to LC/MS/MS analysis on a Micromass Q-ToF-2 mass spectrometer (Micromass, Manchester, UK) coupled with a Waters CapLC capillary HPLC (Waters Corp., USA). This procedure involved separation on a PicoFrit capillary reversed-phase column (5 μ BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm, 15 micron tip [New Objectives, MA, USA]), using a linear water/acetonitrile gradient (0.2% formic acid) , with a 300 micron x 5 mm PepMap C18 column (LC Packings, CA, USA) used as a loading/desalting column. The eluent was introduced directly to the mass spectrometer by electrospray ionization at the tip of the capillary column. Data-dependent MS/MS acquisition was performed on detected peptides with a charge state of 2 or 3. Protein identification from the generated MS/MS data was performed by searching the NCBI non-redundant database using Mascot Daemon (Matrix

Science, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide.

2.3. Results

2.3.1. Morphological symptoms of salinity stress

Two types of salinity stress were imposed: (1) plants were grown for two weeks in the greenhouse under a normal watering regime and then watered with different concentrations of NaCl for an additional six weeks; and (2) seeds were germinated and grown for 7 days in Petri dishes containing solutions of varying NaCl concentrations. While the greenhouse experiments offered a more traditional approach to investigate salinity-induced changes after seeds germinated and plants have attained a specific stage of growth and development (two weeks), the Petri dish experiments offered a more controlled, sterile system where salt is present during other important phases of plant development i.e. germination and seedling growth. The longer-term NaCl-stressed pea plants exhibited symptoms including chlorosis, rolling and drying of leaves at the whole plant level. These symptoms started to appear after four weeks of continuous NaCl stress and increased as the stress continued until five to six weeks. In addition, the above described external symptoms were observed at 75 mM NaCl and were further exacerbated at higher concentrations (Figure 2-1A). The appearance of the roots excised from greenhouse-grown plants stressed with increasing salt concentrations is shown in Figure 2-1B. There was no effect on the root length in plants watered with 25 mM NaCl. However, at higher NaCl concentrations a decrease in fibrosity and overall root growth was observed. In addition the number of root nodules on the roots of plants subjected to salinity were less than on control plants. The roots of seedlings germinated and grown in Petri dishes were not affected at concentrations up to 75 mM NaCl above which there was a visible inhibition of root growth (Figure 2-1C). Based on these morphological symptoms as well as the physical indicators of salinity stress described below 75 mM and 150 mM NaCl were chosen as the two concentrations for proteome analysis.

2.3.2. Physical indicators of salinity stress

In order to choose appropriate concentration(s) of NaCl for our proteome analysis, we measured the lengths and masses of root tissue collected from greenhouse grown plants. The effect of increasing concentrations of NaCl on the mean root length and mass is shown in Figures 2-2A and 2-2B, respectively. The effects of NaCl on mean root length are different from its effect on mean root mass. For example, 75 mM NaCl (as well as those above it) was significantly detrimental to the mean root mass whereas similar concentrations did not negatively affect the root length (Figure 2-2B). This observation is in complete agreement with our observed decrease in root fibrosity as described earlier (Figure 2-1B).

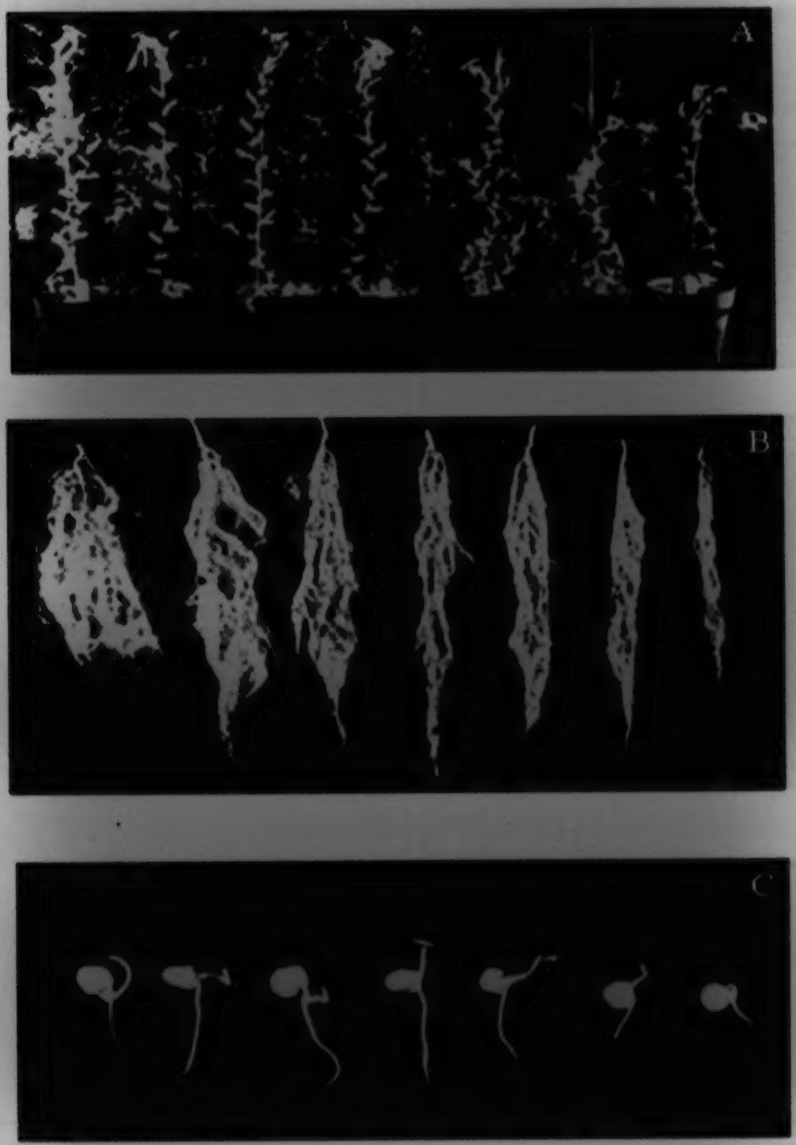


Figure 2-1. Effects of salt stress on pea (A) appearance of plants after eight weeks. Left to right: plants treated with deionised water (control), 25 mM NaCl, 50 mM NaCl, 75 mM NaCl, 100 mM NaCl, 125 mM NaCl and 150 mM NaCl; (B) appearance of roots from plants shown in (A); (C) appearance of seedlings after 7 days of germination in water or salt solutions.

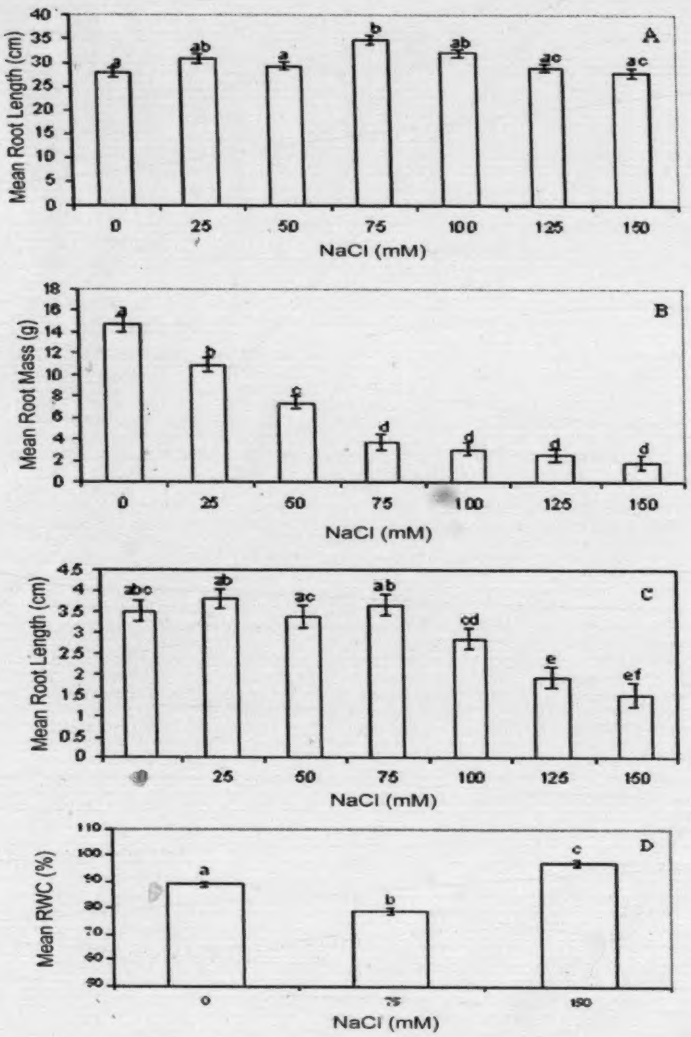


Figure 2-2. Effects of salt stress on root length (A) and mass (B) of plants after eight weeks and root length (C) and relative water content (D) of 7-day-old seedlings germinated in the presence or absence of NaCl. Bars from left to right correspond to: control, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM and 150 mM NaCl-stressed plants. An estimate of least square means and pooled standard errors are presented. Treatments marked by a different letter on the bars for each cluster are significantly different (*P<0.05*).

In the shorter-term salinity stress experiments, we also observed that at concentrations above 75 mM, there was a significant decrease in the mean root length (Figure 2-2C) thus establishing clearly that 75 mM was a crucial concentration of NaCl and could be used in our experiments.

2.3.3. Relative Water Content (RWC)

In addition to the morphological assessment of the salinity-stressed seedlings and plants, we also measured the RWC of root tissues from seedlings germinated in water (control), 75 and 150 mM NaCl as described earlier. RWC measurements after 7 days of continuous germination in the appropriate solutions indicated that the least square mean value of RWC was 89% for control roots, whereas it was 79% for 75 mM NaCl and 97% for the 150 mM NaCl, respectively (Figure 2-2D). The reduction of RWC in the root tissues from seedlings germinated in 75 mM NaCl indicates that water availability to the roots had decreased at this salt concentration as has been observed with numerous plant species in response to salinity and/or osmotic stresses (Chen and Plant, 1999; Jin *et al.*, 2000). Interestingly, the RWC of root tissue isolated from seedlings germinated in 150 mM NaCl was 97% as indicated earlier. This RWC value is significantly ($P<0.05$) higher than measured in the root tissue of control and 75 mM NaCl-treated plants. This may be an indication that, due to irreversible damage as a result of the imposed salinity stress, the root tissues from seedlings at this highest concentration of NaCl used in these studies may have lost their ability to uptake water and revert to the fully turgid state when they are floated for 24 h.

2.3.4. Changes in root proteome

A large number of protein spots extracted from the pea roots were resolved on the gels stained with Coomassie blue followed by silver staining. The proteome profiles shown in Figures 2-3 and 2-4 are quite different and this difference is presumably due to the stage of development of the root tissue used for protein extraction, as well as the growth conditions in the two experimental systems. From a thorough visual analysis of the images of our 2-D gels, we selected 15 spots from the greenhouse root extracts (longer-term, Figure 2-3) and 20 spots from the Petri dish root extracts (shorter-term, Figure 2-4) for identification using Mass Spectrometry (MS). The choice of spots selected for MS analysis was based on the degree of deviation of spot intensity (up- or down-regulation) by visual examination of the gels and their images as well as confirmation of our choice using the spot density/analysis features of the PDQuest software. All the protein spots selected for MS analysis exhibited deviations from the control proteome profile which were reproducible between independent experiments and are indicated in Figures 2-3 and 2-4 by arrows and numbers assigned to each spot. Most of the spots (2-35) were up-regulated in the salinity-treated root tissues, whereas spot 1 was down-regulated in the salinity-stressed tissues (Figure 2-3).

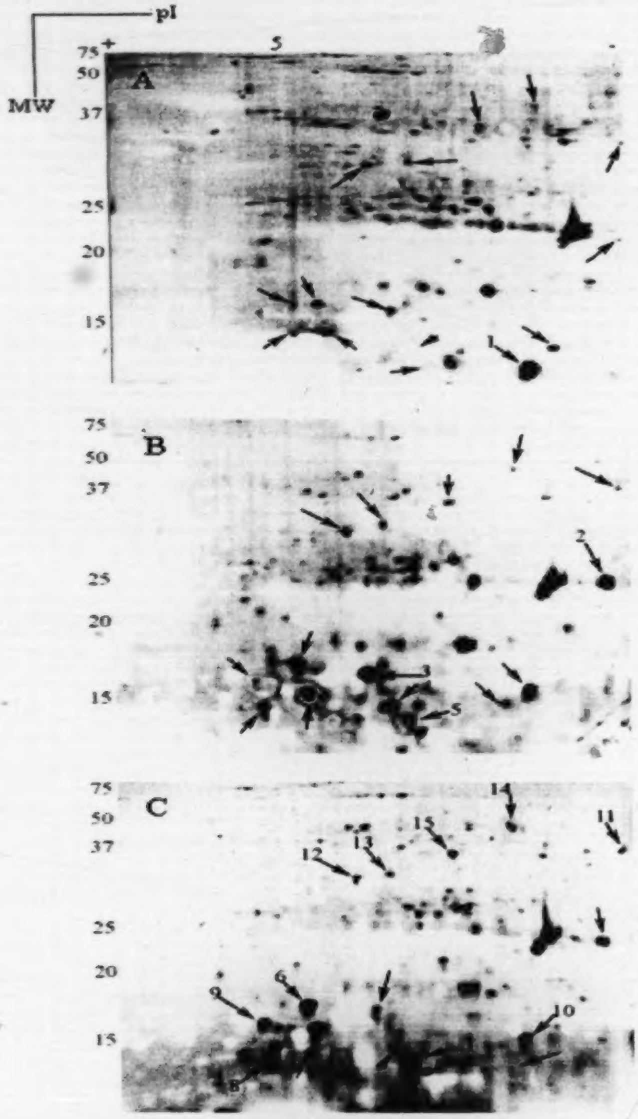
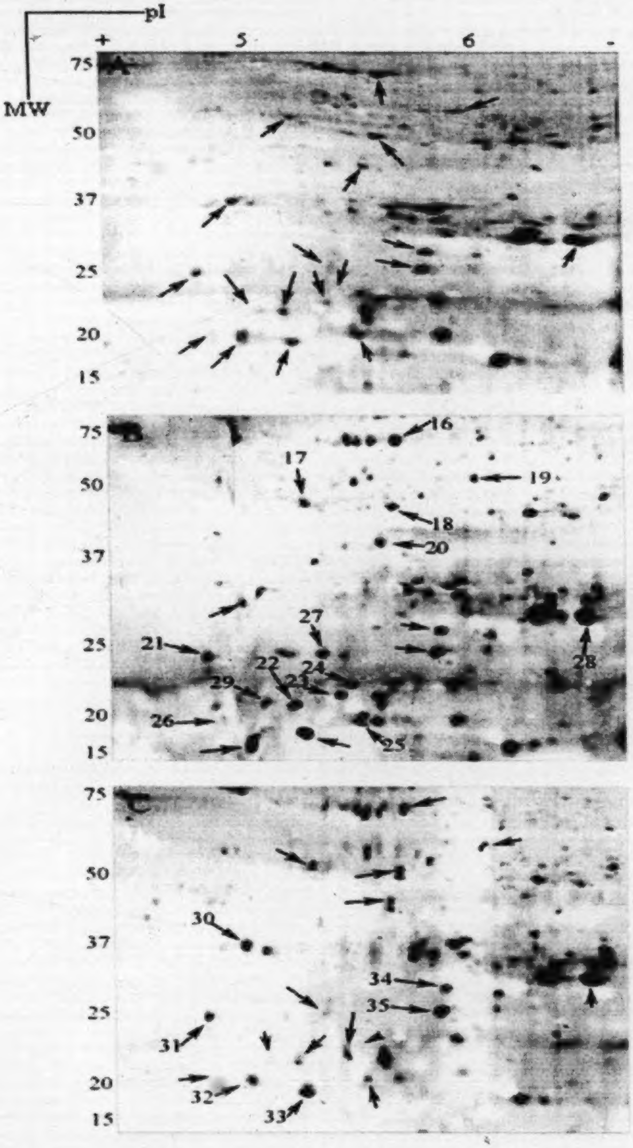

pI
MW
75
50
37
25
20
15
A
5
1
B
2
3
5
C
14
15
11
12
13
9
6
10
8

Figure 2-3. Silver-stained images of total soluble root proteins from roots of control and longer-term salinity-stressed plants grown in the greenhouse separated by two-dimensional electrophoresis (A) control (B) 75 mM NaCl (C) 150 mM NaCl. Protein spots that exhibited deviation in intensity as a result of NaCl treatment are indicated by arrows on all three gels and those that were selected for mass spectrometry by numbers.


pI
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+
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Figure 2-4. Silver-stained images of total soluble root proteins from roots of control seedlings and those germinated in the presence of NaCl (shorter-term stress), separated by two-dimensional electrophoresis (A) control (B) 75 mM NaCl (C) 150 mM NaCl. Protein spots that exhibited deviation in intensity as a result of NaCl treatment are indicated by arrows on all three gels and those that were selected for mass spectrometry by numbers.

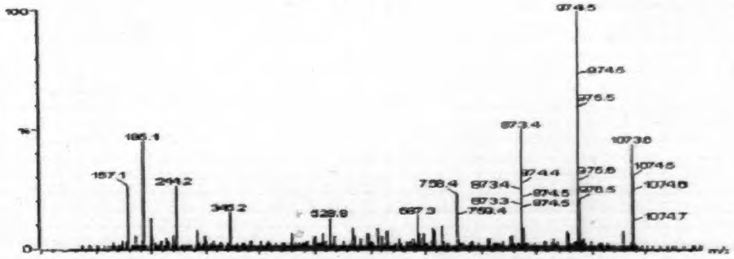
2.3.5. Identities of pea root proteins affected by NaCl stress

The identities of tryptic fragments of selected proteins were established using ESI-Q-TOF MS/MS which generates MS/MS data from which the sequence that rationally fits the observed MS/MS fragmentation pattern is deduced. As an example, the list of fragment ions (Figure 2-5) for spot number 32 is provided. The identities of the proteins as established by this MS/MS analysis are provided in Tables 2-1 and 2-2 for the proteins from greenhouse (longer-term) and Petri dishes (shorter-term).

Among the proteins that were identified, the following were considered to be important with respect to their potential involvement in mediating plant responses to salinity stress: Superoxide Dismutase (SOD; spot number 2, 28), vacuolar ATP-synthase E subunit (spot number 13), isoflavone reductase (spot number 18) as well as the pathogenesis-related proteins that belong to the PR 10 family including abscisic acid (ABA)-responsive protein (ABR)-17 (spot numbers 5, 7, 8, 27, 33); ABR-18 (spot number 25); disease resistance response protein Pi176 (spot numbers 4, 9, 26, 32) and disease resistance response protein Pi49 (spot numbers 9, 32). It is important to note that PR 10 proteins were repeatedly identified in pea root protein extracts as being up-regulated in response to salinity. A closer examination of the locations of these spots on Figures 2-3 and 2-4 indicates the elevated expression of these proteins at both salt concentrations that were used in these studies. Furthermore, the analysis of the spot densities associated with these proteins using the PDQuest software revealed a more than 10-fold increase in the levels of these proteins as a result of the imposed salinity stress.

Start – End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
22 – 33	629.34	1256.67	1256.66	0.01	0	ALVTDADNLTPK
22 – 33	629.34	1256.66	1256.66	-0.00	0	ALVTDADNLTPK
22 – 33	629.33	1256.65	1256.66	-0.01	0	ALVTDADNLTPK
40 – 54	722.88	1443.75	1443.76	-0.00	0	SIEIVEGNGGAGTIK
40 – 54	722.87	1443.73	1443.76	-0.02	0	SIEIVEGNGGAGTIK
56 – 65	569.78	1137.55	1137.56	-0.01	0	LTFVEDGETK
56 – 65	569.78	1137.55	1137.56	-0.00	0	LTFVEDGETK
56 – 65	569.79	1137.56	1137.56	0.00	0	LTFVEDGETK
56 – 65	569.79	1137.56	1137.56	0.01	0	LTFVEDGETK
56 – 65	569.82	1137.62	1137.56	0.06	0	LTFVEDGETK
104 – 115	536.29	1070.57	1070.57	-0.00	0	LSAGPNGGSIAK
104 – 115	536.29	1070.56	1070.57	-0.01	0	LSAGPNGGSIAK
104 – 115	536.29	1070.57	1070.57	-0.00	0	LSAGPNGGSIAK
104 – 115	536.29	1070.57	1070.57	0.00	0	LSAGPNGGSIAK
104 – 115	536.29	1070.57	1070.57	-0.00	0	LSAGPNGGSIAK
124 – 134	572.77	1143.53	1143.54	-0.01	0	GDAAPSEEQLK
124 – 134	572.77	1143.54	1143.54	-0.01	0	GDAAPSEEQLK
124 – 134	572.78	1143.54	1143.54	-0.00	0	GDAAPSEEQLK

(A)



(B)

#	B	b^{++}	b^{*}	b^{*++}	b^{0}	b^{0++}	Seq	y	y^{++}	Y^{*}	Y^{*++}	y^{0}	y^{0++}	#
1	72.04	36.53					**A**							**12**
2	**185.13**	93.07					**L**	1186.63	593.82	1169.61	585.31	1168.62	584.8	**11**
3	**284.20**	142.60					**V**	**1073.55**	537.28	1056.52	**528.76**	1055.54	**528.2**	**10**
4	**385.25**	193.13			367.23	184.12	**T**	**974.48**	487.74	957.45	479.23	956.47	478.7	**9**
5	500.27	250.64			482.26	241.63	**D**	**873.43**	437.22	**856.41**	428.71	855.42	428.2	**8**
6	571.31	286.16			553.30	277.15	**A**	**758.40**	379.71	741.38	371.19	740.39	370.7	**7**
7	686.34	343.67			668.33	334.67	**D**	**687.37**	344.19	670.34	335.67	669.36	335.1	**6**
8	800.38	400.69	783.35	392.18	782.37	391.69	**N**	**572.34**	286.67	555.31	278.16	554.33	277.6	**5**
9	913.46	457.24	896.44	448.72	895.45	448.23	**L**	**458.30**	229.65	441.27	221.14	440.29	220.6	**4**
10	1014.51	507.76	997.48	499.25	996.50	498.75	**T**	**345.21**	**173.11**	328.19	164.60	327.20	164.1	**3**
11	1111.56	556.29	1094.54	547.77	1093.5	547.28	**P**	**244.17**	122.59	227.14	114.07			**2**
12							**K**	147.11	74.06	130.09	65.55			**1**

(C)

Figure 2-5. Representative MS/MS data (A) list of peptides identified in the digestion extract, (B) MS/MS fragmentation spectrum of the peptide with molecular weight 1256.66 which was deconvoluted to generate the sequence ALVTDADNLTPK using the mass of the ions, (C) the ions that were actually observed are indicated in bold.

Another protein of interest with respect to the transduction of stress signals was also identified in this study as nucleoside diphosphate kinase (NDPK; spot number 10). Interestingly, all these proteins are up-regulated in response to the salinity stress (Figures 2-3 and 2-4). The reliability of our approach in identifying novel proteins that are regulated by the imposed stress is supported by the identification of well-characterized proteins involved in salinity stress (e.g. SOD, vacuolar ATP-synthase) and has led to the identification of potentially novel proteins that may play a crucial role in salinity stress responses of the pea (e.g. PR-10 proteins and NDPK). Our discussion below puts our results in context with those of others and further suggests that, upon further validation, the levels of some of these proteins identified may be altered via genetic engineering in order to improve plant stress tolerance.

Table 2-1. Proteins identified by ESI-Q-TOF MS/MS from the roots of longer-term salinity stressed pea plants

Spot #	MS/MS (ESI-Q-TOF)			Identity	[c]Access. No.	Mr /pI
	[a]PM/%	Sequence	[b]% identity			
1	1/3%	NGFTKYTAVGGTASLK	100	hypothetical protein [*Nitrosomonas europaea*]	gi|22954749	44304/ 5.80
2	1/5%	NLAPVSEGGGEPPK	100	superoxide dismutase (EC 1.15.1.1) (Mn) precursor - garden pea [*Pisum sativum*]	gi|279456	26678/ 7.16
3	1/1%	VDEGGAQDK	100	hsc82 protein [*Saccharomyces cerevisiae*]	gi|171723	80823/ 4.76
4	2/15%	LSAGPNGGSIAK GDAAAPTEEQLK	100	Disease resistance response protein Pi176 [*Pisum sativum*]	gi|118931	16967/ 5.07
5	2/11%	LSILEDGK GDAALSDAVR	100	ABA-responsive protein ABR17 [*Pisum sativum*]	gi|1703042	16619/ 5.07
6	2/3%	EQLEALQSETR	100	hypothetical protein [*Cytophaga hutchinsonii*]	gi|23138320	31932/ 4.90
7	34/49%	GVFVFDDEYVSTVAPPK DADEIVPK EAQGVEIIEGNGGPGTIK KLSILEDGK LSILEDGK GDAALSDAVR GDAALSDAVRDETK AIEGYVLANPGY	100	ABA-responsive protein ABR17 [*Pisum sativum*]	gi|1703042	16619/ 5.07
8	16/41%	GVFVFDDEYVSTVAPPK DADEIVPK EAQGVEIIEGNGGPGTIK LSILEDGK GDAALSDAVR GDAALSDAVRDETK	100	ABA-responsive protein ABR17 [*Pisum sativum*]	gi|1703042	16619/ 5.07
9	2/13%	ALVTDADNLTPK LTFVEDGETK	100	Disease resistance response protein Pi49 (PR 10) [*Pisum sativum*]	gi|118933	16794/ 4.94
	3/21%	ALVTDADTLTPK LTFVEDGETK GDAAAPTEEQLK	100	Disease resistance response protein Pi176 [*Pisum sativum*]	gi|118931	16967/ 5.07
10	7/20%	IIGATNPAQSEPGTIR NVIHGSDAVESANK	100	Nucleoside diphosphate kinase I (NDK I) (NDP kinase I) (NDPK I) (P18) [*Pisum sativum*]	gi|1346672	16452/ 5.94
11	7/23%	GYATEPVPER VAILGAAGGIGQPLSLLMK ALEGADVVIIPAGVPR DDLFNINAGIVK LFGVTTLDVVR TQDGGTEVVTAK	100	malate dehydrogenase (EC 1.1.1.37) precursor – alfalfa [*Medicago sativa*]	gi|7431177	36003/ 8.80
12	1/2%	IAANMIGQDR	100	COP8 (constitutive	gi|21592680	45182/

				photomorphogenic) homolog [*Arabidopsis thaliana*]		4.87
13	3/8%	ELIVQSLLR IVFENTLDAR	100	Vacuolar ATP synthase subunit E (V-ATPase E subunit) (Vacuolar proton pump E subunit) [*Mesembryanthemum crystallinum*]	gi|3334410	26318/ 6.52
14	1/1%	VAAISVPR	100	AGR_C_2215p [*Agrobacterium tumefaciens* str. C58 (Cereon)]	gi|15888538	55519/ 6.10
15	1/1%	ALALEDLR	100	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Lythrum hyssopifolia*]	gi|168335	53337/ 6.22

Note: [a] Number of peptides matched/ sequence percentage coverage.

[b] Percentage identity between the amino-acids present in MS/MS tag and the sequences in databases.

[c] Accession number is Mascot search result using NCBI and other databases.

Table 2-2. Proteins identified by ESI-Q-TOF MS/MS from the roots of shorter-term salinity stressed pea plants

Spot No	MS/MS (ESI-Q-TOF)			Identity	[c]Access. No.	Mr /pI
	[a]PM/%	Sequence	[b]% identity			
16	7/ 9%	DGGSDYLGK AVDNVNTIIAPALIGK HIANLAGNK YNQLLR	100	Enolase [*Lupinus luteus*]	gi|6996529	47926/ 5.14
17	1/ 3%	NVVVSLTNATSTTHK	100	putative protein At5g53750.1 [*Arabidopsis thaliana*]	gi|15238832	46654/ 6.25
18	9/18%	AGNPTYALVR LTEAANPETK QVDTVICAAGR HDAVEPVR AANDPNTLNK GDAVYEIDPAK	100	Isoflavone reductase (IFR) (NADPH:isoflavone oxidoreductase) [*Pisum sativum*]	gi|1708427	35578/ 5.39
19	13/28%	DELDIVIPTIR VPEGFDYELYNR ASCISFK YIYTIDDDCFVAK DPTGHEINALEQHIK FVDAVLTIPK VICDHLGYGVK ASNPFVNLK CYIELSK LGTIDPYFIK	100	reversibly glycosylatable polypeptide 1 - garden pea [*Pisum sativum*]	gi|7488840	42059/ 5.73
20	4/7%	VLQAQDDLVNK QIQQMVR	100	(U84268) YLP [*Hordeum vulgare* subsp. vulgare]	gi|4099148	26359/ 6.57
21	1/5%	LQEQPPFDK	100	Translationally controlled tumor protein homolog (TCTP) [*Pisum sativum*]	gi|2507443	18943/ 4.71
22	1/5%	TETTSFLITK	100	Pea Lectin, Chain A [*Pisum sativum*]	gi|230612	19973/ 5.31
23	1/3%	VLVVDDEDR	100	similar to two-component response regulator (ResD) [*Listeria innocua*]	gi|16801128	27520/ 5.11
24	1/0%	LIIEVQVK	100	hypothetical protein At2g42470.1 [*Arabidopsis thaliana*]	gi|15227946	103863/ 6.43
25	1/7%	LFEGPNGGSVGK	100	ABA-responsive protein ABR18 [*Pisum sativum*]	gi|1703043	17013/ 5.25
26	5/22%	ALVTDADTLTPK LSAGPNGGSIAK GDAAAPTEEQLK	100	Disease resistance response protein Pi176 [*Pisum sativum*]	gi|118931	16967/ 5.07
27	1/6%	GDAALSDAVR	100	ABA-responsive protein ABR17 [*Pisum sativum*]	gi|1703042	16619/ 5.07
28	2/9%	NLAPVSEGGGEPPK HASEVYEK	100	superoxide dismutase (EC 1.15.1.1) (Mn) precursor – garden pea	gi|279456	26678/ 7.16

				[*Pisum sativum*]		
29	1/3%	LINAEDATVPR	100	hypothetical protein [*Thermobifida fusca*]	gi|23018931	31531/ 6.05
30	1/2%	AGGGLVAVSGDDHR	100	adenine deaminase [*Methanothermobacter thermautotrophicus*]	gi|15678886	58132/ 4.75
31	1/1%	AGEAQGWR	100	Molecular chaperone [*Rhodospirillum rubrum*]	gi|22965455	73310/ 5.37
32	18/37%	LSAGPNGGSIAK LTFVEDGETK GDAAPSEEQLK ALVTDADNLTPK SIEIVEGNGGAGTIK	100	Disease resistance response protein Pi49 (PR 10) [*Pisum sativum*]	gi|118933	16794/ 4.94
	20/38%	ALVTDADTLTPK SIEIVEGNGGAGTIK LTFVEDGETK LSAGPNGGSIAK GDAAAPTEEQLK	100	Disease resistance response protein Pi176 [*Pisum sativum*]	gi|118931	16967/ 5.07
33	10/19%	DADEIVPK KLSILEDGK LSILEDGK GDAALSDAVR GDAALSDAVRDETK	100	ABA-responsive protein ABR17 [*Pisum sativum*]	gi|1703042	16619/ 5.07
34	1/1%	LRAEGTLVSPR	100	hypothetical protein [*Azotobacter vinelandii*]	gi|23102685	73007/ 5.81
35	1/1%	GNDGILYAGSK	100	Ser/Thr protein kinase [*Caenorhabditis elegans*]	gi|16904242	110437/ 6.98

Note: [a] Number of peptides matched/ sequence percentage coverage.

[b] Percentage identity between the amino-acids present in MS/MS tag and the sequences in databases.

[c] Accession number is Mascot search result using NCBI and other databases.

2.4. Discussion

Many studies on plant-abiotic stress interactions have demonstrated the utility of proteomics research in identifying novel stress-responsive proteins for subsequent genetic improvement of stress tolerance (Dubey and Grover, 2001). Similar approaches, based on two-dimensional electrophoretic separation of proteins have also been used to investigate root (Majoul *et al.*, 2000) and shoot (Ouerghi *et al.*, 2000) proteome-level differences between varieties of wheat exhibiting differential salinity tolerance. The results from our current study led to the identification of 35 root proteins whose levels are altered as a result of salinity. Some of the proteins identified have been well characterized in terms of their response to salinity and other abiotic stresses and further validate the utility of a similar approach in applied plant biology. Other proteins have not been well-studied with respect to their role in abiotic stress responses in pea, but have been with respect to biotic stresses and may represent those with a common role.

The family of SODs are among the cell's first line of defence against reactive oxygen species (ROS) generated as a result of various abiotic and biotic stresses and their main function is to eliminate the superoxide radicals from the cell (Grene, 2002; Hernandez *et al.*, 2002). Three types of SODs (EC 1.15.1.1) are present in plants: iron-SOD (Fe-SOD), manganese-SOD (Mn-SOD), and copper-zinc SOD (Cu-Zn SOD) and are located in different plant sub-cellular organelles (Grene, 2002). Increased activities of several antioxidant enzymes in the leaf mitochondria of salt-tolerant pea cultivars under salt stress (Gomez *et al.*, 1999; Hernandez *et al.*, 2000) and drought

(Wu *et al.*, 1999) have been reported. In the NaCl-tolerant variety, the activity of mitochondrial Mn-SOD is increased, while that of Cu-Zn SOD remains constant, whereas in NaCl-sensitive plants, Cu-Zn SOD activity decreases by about 35% (Hernandez *et al.*, 2000). Our results supports a crucial role for SOD in mediating salinity stress responses and further validates the utility of proteomics-based approaches for identifying proteins involved in mediating plant responses to stress.

Three primary proton pumps maintain the intracellular trans-membrane proton gradient in plant cells, including P-type ATPases, H^+-pumping pyrophosphatases (H^+-PP_iases) and vacuolar-type H^+-pumping ATP hydrolase (H^+-ATPase, V-ATPase, VHA; Kluge *et al.*, 2003). V-ATPase, a housekeeping enzyme and the major H^+-pump of the tonoplast, maintains cytosolic ion homeostasis during cellular metabolism and helps cope with environmental and metabolic changes by modulating its activity and the transcription of various subunits of V-ATPase is up-regulated by salinity in various plants (Ratajczak, 2000). One of the mechanisms involved in the adaptation of plants to salinity stress is the efficient exclusion of excess sodium from the cytoplasm into the vacuole (Dietz *et al.*, 2001). The accumulation of sodium ions in the vacuole is thought to be mediated by a tonoplast Na^+/H^+ antiporter that can utilize the proton-motive force generated by the vacuolar H^+-ATPase (Apse *et al.*, 1999; Dietz *et al.*, 2001). Furthermore, the main strategy of salt-tolerance in *Suaeda salsa* seemed to be an up-regulation of V-ATPase activity, due to an increase in the levels of V-ATPase (Wang *et al.*, 1999).

Isoflavone reductase (IFR) is a NADPH-dependent enzyme involved in the biosynthesis of defence-related isoflavonoid phytoalexins (Oommen *et al.*, 1994). IFR has been purified and characterized from several leguminous species and cDNA clones for pea IFR have been isolated (Paiva *et al.*, 1994), and due to its role in phytoalexin biosynthesis, is thought to play a critical role in mediating plant responses to pathogens; however, its role in mediating abiotic stress response is not clear. IFR can be induced by wounding and UV radiation in addition to pathogen infection in grapefruit (Lers, 1998). In maize seedlings, under sulphur starvation conditions an IFR-like protein with >70% homology to IFR, is persistently induced, which correlates with a decline in cellular glutathione levels (Petrucco *et al.*, 1996). These observations led the authors to suggest a possible link between glutathione and IFR-like protein and that IFR (IFR-like proteins) plays a crucial role in restoring NADPH levels when the levels of this coenzyme are insufficient to meet cellular oxidative demands, particularly under abiotic or biotic stress (Petrucco *et al.*, 1996). Further investigation of *IFR* gene expression in response to abiotic stresses may provide insights into the mechanisms underlying the coordinated transcriptional activation of a complex secondary biosynthetic pathway and its role in cellular defence (Oommen *et al.*, 1994).

Apart from the proteins discussed above, a group of pathogenesis-related proteins (PR 10 proteins) were repeatedly identified in the protein extracts of shorter- as well as longer-term salinity-stressed root tissues. Pathogenesis related (PR) proteins are plant proteins that are induced in response to various stress conditions. PR proteins occur as small multigene families in various species, are expressed during development in a tissue-specific manner, are generally localized in the apoplast and vacuolar compartment, and are differentially induced by endogenous and exogenous signalling compounds suggesting that they may have important functions beyond a response to pathogens (Van Loon and Van Strien, 1999). The intracellular pathogenesis-related (IPR or PR 10) proteins are a family of small homologous, primarily acidic proteins, which have been identified in various plants. Usually these proteins accumulate due to fungal and bacterial infection, but they may also be regulated by stress factors, such as drought, wounding and darkness, or may be developmentally regulated in certain tissues (Osmark *et al.*, 1998).

Optimal alignment of *Pi49* and *Pi176* cDNA (disease resistance response genes of the *PR 10* family; *PR 10.1* and *10.2*, respectively) revealed an overall identity of 95% between these two genes, suggesting that these genes may be part of a multigene family (Fristensky *et al.*, 1988). In addition to *Pi49* and *Pi176*, two other genes encoding proteins induced by ABA and designated as ABA-responsive proteins *ABR17* and *ABR18* have been identified and designated as *PR 10.4* and *10.5*, respectively (Iturriaga *et al.*, 1994). Our current study identified several members of the PR 10 protein family as being increased in the roots of pea plants (spots 25, 26, 27, 32, 33 and 4, 5, 7, 8, 9 in the shorter-term and longer-term stress, respectively). Interestingly, Chen *et al.* (1999), as well as Jin *et al.* (2000), performed 2-D electrophoretic analysis of proteins synthesized *de novo* upon salinity stress, which revealed the presence of proteins that appear to be in the same molecular weight and pI range as some of the PR 10 proteins identified in our current study. However, the identities of the proteins elevated in response to salinity stress were not established by Chen *et al.* (1999) or Jin *et al.* (2000) and therefore, this constitutes the first report of up-regulation of pathogenesis related proteins (PR 10) due to salt stress in pea roots.

Despite the identification of several proteins belonging to the PR 10 family from several species and their association with pathogenesis as well as abiotic stresses in some species, the biological function of PR 10 proteins has not been clearly established and remains controversial. For example, PR 10 proteins from ginseng have been shown to possess ribonuclease (RNase) activity (Moiseyev *et al.*, 1997) that may potentially be involved in defence against pathogens. The high level of PR 10 proteins in roots may also be part of a constitutive defensive mechanism in the plant organ most exposed to environmental stress, but high levels of PR 10 proteins in other tissues as well as their varied expression patterns causes doubt about the RNase hypothesis (Biesiadka *et al.*, 2002). Another possible role for PR 10 proteins as cytokinin-binding proteins (CSBP) has been proposed as a result of the successful docking of zeatin in the putative active site of PR 10 from yellow lupin whose three-dimensional structure has been determined at a high resolution (Biesiadka *et al.*, 2002).

Even though the biological activities associated with the various members of the PR 10 protein family still remains unclear, a possible relationship between PR 10 proteins and salinity stress response has been revealed in rice (Moons *et al.*, 1997). This study demonstrated that jasmonic acid induces the expression of PR 1 and PR 10, as well as the salt-responsive SalT protein in rice roots, whereas ABA has antagonistic effects. Furthermore, induction of *PR 10* gene expression by ABA in pea (Iturriaga *et al.*, 1994) and cytokinins in periwinkle callus (Carpin *et al.*, 1998) as well as jasmonate and salicylate in tomato has been demonstrated and correlates with chilling tolerance (Ding *et al.*, 2002). These results suggest that there may be an intricate relationship between the levels of various phytohormones and *PR 10* gene expression with relevance to stress tolerance in plants. Furthermore, the overexpression of a mitogen activated protein kinase in rice (*OsMAPK5*) negatively modulates *PR 10* gene expression with a concomitant increase in tolerance to drought, salinity and cold temperature stresses in the transgenic plants (Xiong and Yang, 2003). In addition, Borsics and Lados (2002) demonstrated that a large variety of environmental stresses including salinity induces the expression of *PR 10* genes in the model legume alfalfa. These studies further support the hypothesis that the coordinated expression of *PR 10* genes, probably regulated by the action of phytohormones and specific protein kinases may play a critical role in mediating plant responses to abiotic and biotic stresses.

In addition to the various members of the PR 10 protein family that were induced by salinity stress in pea, the identification of another protein that was up-regulated in response to salinity, nucleoside diphosphate kinase (NDPK), further supports a potentially crucial role for PR 10 proteins in mediating salinity responses. NDPK is a housekeeping enzyme that maintains the intracellular levels of all (d)NTPs except ATP, and may play a significant role in signal transduction pathways (Otero, 2000). In plants, NDPK isoforms are involved in the phytochrome B response, UV-B light signalling, hormone response as well as heat stress (Escobar Galvis *et al.*, 2001). Furthermore, the constitutive overexpression of *AtNDPK2* in *Arabidopsis* down-regulates the accumulation of ROS and enhances tolerance to multiple stresses through the coordination of NDPK2 activity with MAPK-mediated H_2O_2 signalling (Moon *et al.*, 2003). The elevated levels of key proteins associated with ROS-mediated signal transduction that involves NDPK and MAPK and PR 10 proteins could, therefore, be a novel response to salinity stress in peas and perhaps other plant species. The precise function of the PR 10 proteins in normal and stress physiology of plants is unclear and the elucidation of the role(s) of these proteins, as well as their interaction with or regulation by NDPK and MAP kinases, may further our understanding of the stress-induced signal transduction cascades in plants.

Despite decades of extensive research into understanding plant responses to abiotic stresses, the efforts of plant breeders to improve salinity stress tolerance in plants have been largely less successful due to the complexity of the responses involved (Zhu, 2002). A detailed understanding of the various signal transduction pathways as well as the regulatory mechanisms that underlie specialized gene expression during salinity responses, may one day pave the way for rational crop improvement. Our proteomics-based investigation in pea has, for the first time, revealed the potential involvement of PR 10 proteins and NDPK in mediating salinity stress responses in pea roots as part of a potentially novel signal transduction pathway involving SOD-H_2O_2, NDPK, MAPK and PR 10 proteins. We are currently in the process of characterizing the temporal and spatial expression patterns of the various members of the PR 10 protein family as well as NDPK under normal growth and development of the pea and in response to various stresses in order to further delineate this signal transduction pathway.

2.5. References

Anon. (1989) SAS/STAT User.s Guide Version 6, Fourth Edn. Cary, NC, USA: SAS Institute.

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science **285:** 1256–1258.

Biesiadka, J, Bujacz, G, Sikorski, M.M. and Jaskolski, M. (2002) Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. J Mol Biol **319:** 1223–1234.

Borsics, T. and Lados, M. (2002) Dodder infection induces the expression of a pathogenesis-related gene of the family PR-10 in alfalfa. J Exp Bot **53:** 1831–832.

Carpin, S., Laffer, S., Schoentgen, F., Valenta, R., Chenieux, J.-C., Rideau, M. and Hamdi, S. (1998) Molecular characterization of a cytokinin-inducible periwinkle protein showing sequence homology with pathogenesis-related proteins and the Betv 1 allergen family. Plant Mol Biol **36:** 791–798.

Chen, C.C.S. and Plant, A.L. (1999) Salt-induced protein synthesis in tomato roots: the role of ABA. J Exp Bot **50:** 677–687.

Damerval, C., de Vienne, D., Zivy, M. and Thiellement, H. (1986) Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. Electrophoresis **7:** 53–54.

Dietz, K.J., Tavakoli, N., Kluge, C., Mimura, T., Sharma, S.S., Harris, G.C., Chardonnens, A.N. and Golldack, D. (2001) Significance of the V-type ATPase for the adaptation to stressful growth conditions and its regulation on the molecular and biochemical level. J Exp Bot **52:** 1969–1980.

Ding, C.-K., Wang, C.Y., Gross, K.C. and Smith, D.L. (2002) Jasmonate and salicylate induce the expression of pathogenesis-related protein genes and increase resistance to chilling injury in tomato fruit. Planta **214:** 895–901.

Dubey, H. and Grover, A. (2001) Current Initiatives in proteomics research: the plant perspective. Current Science **80:** 262–269.

Escobar Galvis, M.L., Marttila, S., Hakansson, G., Forsberg, J. and Knorpp, C. (2001) Heat stress response in pea involves interaction of mitochondrial nucleoside diphosphate kinase with a novel 86-kilodalton protein. Plant Physiol **126:** 69–77.

Flowers, T.J. and Yeo, A.R. (1995) Breeding for salinity resistance in crop plants: where next? Aust J Plant Physiol **22:** 875–884.

Fristensky, B., Horovitz, D. and Hadwiger, L.A. (1988) cDNA sequence for pea disease resistance response genes. Plant Mol Biol **11:** 713–715.

Gomez, J.M., Hernandez, J.A., Jimenez, A., del Rio, L.A. and Sevilla, F. (1999) Differential response of antioxidative enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plants. Free Rad Res **31:** S11-S18.

Grene, R. (2002) Oxidative stress and acclimation mechanisms in plants. The *Arabidopsis* Book, pp.1-19. American Society of Plant Biologists.

Greenway, H. and Munns, R. (1980) Mechanisms of salt tolerance in non-halophytes. Ann Rev Plant Physiol **31:** 149–190.

Gygi, S.P. and Aebersold, R. (2000) Mass spectrometry and proteomics. Curr Opin Chem Biol **4:** 489-494.

Gygi, S.P., Rochon, Y., Franza, B.R. and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol **19:** 1720-1730.

Hasegawa, P.M., Bressan, R.A., Zhu, J.-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. Ann Rev Plant Physiol Plant Mol Biol **51:** 463–499.

Hernandez, J.A. and Almansa, M.S. (2002) Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. Physiologia Plantarum **115:** 251–257.

Hernandez, J.A., Jimenez, A., Mullineaux, P. and Sevilla, F. (2000) Tolerance of pea (*Pisum sativum* L.) to long-term stress is associated with induction of antioxidant defenses. Plant Cell Env **23:** 853–862.

Iturriaga, E.A., Leech, M.J., Barratt, D.H.P. and Wang, T.L. (1994) Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. Plant Mol Biol **24:** 235–240.

Jin, S., Chen, C.C.S. and Plant, A.L. (2000) Regulation by ABA of osmotic-stress-induced changes in protein synthesis in tomato roots. Plant Cell Environ **23:** 51–60.

Kluge, C., Lahr, J., Hanitzsch, M., Bolte, S., Golldack, D. and Dietz, K.-J. (2003) New insight into the structure and regulation of the plant vacuolar H^+-ATPase. J Bioenergetics and Biomem **35:** 377–388.

Lers, A., Burd, S., Lomaniec, E., Droby, S. and Chalutz, E. (1998) The expression of a grapefruit gene encoding an isoflavone reductase-like protein is induced in response to UV irradiation. Plant Mol Biol **36:** 847–856.

Majoul, T., Chahed, K., Zamiti, E., Ouelhazi, L. and Ghrir, R. (2000) Analysis by two-dimensional electrophoresis of the effect of salt stress on the polypeptide patterns in roots of a salt-tolerant and a salt-sensitive cultivar of wheat. Electrophoresis **21:** 2562–2565.

Moiseyev, G.P., Fedoreyeva, L.I., Zhuravlev, Y.N., Yasnetskaya, E., Jekel, P.A. and Beintema, J.J. (1997) Primary structures of two ribonucleases from ginseng calluses. New members of the PR 10 family of intracellular pathogenesis-related plant proteins. FEBS Lett **407:** 207–210.

Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.-S., Kim, D.H., Nam, J., Bahk, J., Hong, J.C., Lee, S.Y., Cho, M.J., Lim, C.O. and Yun, D.J. (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proc Natl Acad Sci USA **100:** 358–363.

Moons, A., Prinsen, E., Bauw, G. and Van Montagu, M. (1997) Antagonistic effects of Abscisic acid and Jasmonates on salt-stress-inducible transcripts in rice roots. Plant Cell **9:** 2243–2259.

Oommen, A., Dixon, R.A. and Paiva, N.L. (1994) The elicitor-inducible alfalfa isoflavone reductase promoter confers different patterns of developmental expression in homologous and heterologous transgenic plants. Plant Cell **6:** 1789–1803.

Osmark, P., Boyle, B. and Brisson, N. (1998) Sequential and structural homology between intracellular pathogenesis-related proteins and a group of latex proteins. Plant Mol Biol **38:** 1243–1246.

Otero, A.S. (2000) NM23/nucleoside diphosphate kinase and signal transduction. J Bioenergetics and Biomem **32:** 269–275.

Ouerghi, Z., Remy, R., Ouelhazi, L., Ayadi, A. and Brulfert, J. (2000) Two-dimensional electrophoresis of soluble leaf proteins, isolated from two wheat species (*Triticum durum* and *Triticum aestivum*) differing in sensitivity towards NaCl. Electrophoresis **21:** 2487–2491.

Paiva, N.L., Sun, Y., Dixon, R.A., Van Etten, H.D. and Hrazdina, G. (1994) Molecular cloning of isoflavone reductase from pea (*Pisum sativum* L.): evidence for a 3R-isoflavanone intermediate in (+)-pisatin biosynthesis. Arch Biochem Biophy **312:** 501–510.

Petrucco, S., Bolchi, A., Foroni, C., Percudani, R., Rossi, G.L. and Ottonello, S. (1996) A maize gene encoding an NADPH binding enzyme highly homologous to isoflavone reductases is activated in response to sulfur starvation. Plant Cell **8:** 69–80.

Pitman, M.G. and Lauchli, A. (2002) Global impact of salinity and agricultural ecosystems. In Salinity: Environment-Plants-molecules, pp. 3-20. Eds A Lauchli and U Luttge. Massachusetts: Kluwer Academic Publishers.

Ratajczak, R. (2000) Structure, function and regulation of the plant vacuolar H^+-translocating ATPase. Biochim Biophys Acta **1465:** 17–36.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. and Bennett, J. (2002) Proteomic analysis of rice leaves during drought stress and recovery. Proteomics **2:** 1131–1145.

Shi, H. and Zhu, J.-K. (2002) Regulation of expression of the vacuolar Na^+/H^+ antiporter gene AtNHX1 by salt stress and abscisic acid. Plant Mol Biol **50:** 543–550.

Van Loon, L.C. and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR1 type proteins. Physiol Mol Plant Pathol **55:** 85–97.

Wang, Y., Nowak, G., Culley, D., Hadwiger, L.A. and Fristensky, B. (1999) Constitutive expression of pea defense gene DRR 206 confers resistance to Blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). Mol Plant-Microbe Inte **12:** 410–418.

Wang, H., Miyazaki, S., Kawai, K., Deyholos, M., Galbraith, D.W. and Bohnert, H.J. (2003) Temporal progression of gene expression responses to salt shock in maize roots. Plant Mol Biol **52:** 873–891.

Wu, G., Wilen, R.W., Robertson, A.J. and Gusta, L.V. (1999) Isolation, chromosomal localization, and differential expression of mitochondrial manganese superoxide dismutase and chloroplastic copper/zinc superoxide dismutase genes in wheat. Plant Physiol **120:** 513–520.

Xiong, L. and Zhu, J.-K. (2002) Salt tolerance. The *Arabidopsis* Book, pp.1-24. American Society of Plant Biologists.

Xiong, L. and Yang, Y. (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid–inducible mitogen-activated protein kinase. The Plant Cell **15:** 745–759.

Zivy, M. and de Vienne, D. (2000) Proteomics: a link between genomics, genetics and physiology. Plant Mol Biol **44:** 575–580.

Zhu, J.-K. (2002) Salt and drought stress signal transduction in plants. Ann Rev Plant Biol **53:** 247–273.

3. Constitutive expression of a PR 10 protein enhances the germination of *Brassica napus* L. under saline conditions

3.1. Introduction

Salinity negatively affects crop production worldwide through numerous deleterious effects on plant cells including membrane disorganization, generation of toxic metabolites and reactive oxygen species, as well as the inhibition of photosynthesis (Hasegawa *et al.*, 2000). Plants respond to salinity by activating a varied and complex set of defense pathways that invoke several responses ultimately culminating in tolerance or susceptibility, making the generation of salinity-tolerant crops through classical breeding difficult (Zhu, 2002). On the other hand transgenic approaches, including the enhanced expression of a Na^+/H^+ antiporter, have been successful (Apse *et al.*, 1999), and the completion of plant genome projects, rapid development of post-genomic techniques including microarrays and proteomics-based strategies may identify additional targets for engineering salinity tolerance.

We employed a strategy using two-dimensional electrophoresis and mass spectrometry (MS) to characterize proteome-level changes in the roots of pea (*Pisum sativum* L.) plants in response to salinity (Kav *et al.*, 2004). These studies revealed a significant salinity-induced increase in the levels of several members of a PR protein family (PR 10).

A version of this chapter has been published: ***Srivastava S.****, Fristensky B. and Kav N.N.V. (2004) Constitutive expression of a PR 10 protein enhances the germination of Brassica napus under saline conditions,* ***Plant and Cell Physiology****, 45, 1320-1324.*

Other researchers have also shown that PR 10 proteins are expressed in response to pathogen infection as well as abiotic stress such as drought and salinity (Dubos and Plomion, 2001; Park *et al.*, 2004, Moon *et al.*, 2003). PR 10 proteins are encoded by a gene family and have been characterized from various plant species (Liu *et al.*, 2003). While *PR 10* genes were originally identified in peas expressing resistance to fungi (Riggleman *et al.*, 1985), PR 10 has also been described as responding to stress and abscisic acid, as a pollen allergen and has been shown to be constitutively expressed in roots (see refs. in Tewari *et al.*, 2003[a]). The precise role of PR 10 in these processes has remained elusive although PR 10 proteins from ginseng, white lupin and hot pepper have been demonstrated to possess RNase activities (Moiseyev *et al.*, 1994; Bantignies *et al.*, 2000; Park *et al.*, 2004). However, molecular docking experiments have also suggested that a PR 10 protein from white lupin may be able to bind cytokinins (Biesiadka *et al.*, 2002), although this remains to be verified experimentally. With respect to disease resistance expression of pea *PR 10.1* in potato conferred resistance to early dying disease (Chang *et al.*, 1993), but constitutive expression of this gene in transgenic canola does not enhance resistance to blackleg (Wang *et al.*, 1999).

Our observation that pea PR 10 proteins are significantly induced upon salinity stress led us to hypothesize that these proteins may have a significant role in protecting plant cellular components from the deleterious effects of salinity (Kav *et al.*, 2004). Transgenic *Brassica napus* L. lines constitutively expressing the pea *PR 10.1* gene were generated to evaluate the utility of this gene in engineering resistance to fungal diseases in *B. napus* (Wang *et al.*, 1999) and the availability of these plants allowed us to quickly test our hypothesis. Furthermore, *B. napus* (canola) is an economically important agricultural commodity and increasing its ability to germinate and survive under water deficit conditions is highly desired. The results presented here indicate that these transgenic plants constitutively expressing pea *PR 10.1* gene are able to germinate and develop better in the presence of NaCl when compared to untransformed control plants. Our findings therefore support our hypothesis that at least one member of the pea PR 10 protein family may have a vital role in protecting plants from abiotic stresses such as salinity.

3.2. Materials and methods

Brassica napus L. cv. Westar seeds (untransformed and seeds from T_2 plants of a transgenic line GN1-5#22; Wang *et al.,* 1999) were surface sterilized for 1 min in 70% ethanol and 5 min in 5 % (v/v) sodium hypochlorite in water. Seeds were rinsed with sterile deionized water 3-4 times and were placed on a sterile filter paper in a Petri dish containing five mL of deionized water (control) or 75 mM NaCl solution. The experiment was designed in a randomized complete block design (RCBD) and consisted of five seeds per Petri dish and a total of five replicates for both the untransformed control and transgenic seeds at 0 and 75 mM NaCl. The plates were sealed with laboratory film and incubated in the dark at room temperature (20±2°C) for 7 days at which time root and shoot lengths were measured. The data were analyzed using the GLM procedure of SAS (Statistical Analysis System, 1985).

Root and shoot tissues from 7-day-old seedlings were pulverized under liquid nitrogen. Total RNA extraction and subsequent DNase treatment were done using the RNeasy Plant Mini Kit and the RNase-free DNase set, respectively (QIAGEN, Ontario, Canada). Total RNA (50 ng) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Hercules, CA, USA). PCR reactions using the newly synthesized cDNA (2 μL) as the template were carried out using primers specific to the pea PR 10.1 protein coding sequence (5'-CTAGTTACAGATGCTGATAAC-3' and 5'-CATCCCCCTTAGCTTTG TCAG-3') using a PCR Master Mix (Promega, MD, USA). PCR amplification steps consisted of a preliminary denaturation step at 94 °C for 2 min, followed by 35 cycles of 1 min steps at 94°C, 62°C and 72°C, performed on a Gene Amp PCR System 9700 (Applied Biosystems, CA USA). Plant *18S rRNA* primers (Duval *et al.*, 2002) were used as an internal control. PCR products were run on 1% agarose gel and visualized under UV light after staining with ethidium bromide.

Shoot tissue from control and transgenic seeds germinated and grown for 7 days in Petri dishes in the presence or absence of NaCl (75 mM) were ground in liquid nitrogen to a fine powder. The homogenized tissue (0.1 g) was resuspended in 1 mL ice-cold 10% w/v trichloroacetic acid (TCA), 0.07% w/v dithiothreitol (DTT) – acetone solution and incubated at -20°C for 1 h. Samples were centrifuged at 13,000 rpm for 15 min, and the supernatants discarded. The pellets were resuspended in 1 mL ice-cold acetone containing 0.07% w/v DTT and incubated at -20°C for 1 hour. The samples were centrifuged as before and the wash step was repeated twice. Following the final wash step, the pellets, which contained the precipitated proteins, were dried for 15 min at room temperature and resolubilized with 0.5 mL rehydration buffer containing 5 μL tributylphosphine (TBP, Bio Rad, Mississauga, Canada). Samples were vortexed vigorously and incubated at 4°C overnight after which they were centrifuged as before. The supernatants were collected and the protein concentrations were determined using a modified Bradford assay (Bio Rad) with bovine serum albumin (BSA; Pierce Biotechnology Inc., Rockford, USA) as the standard.

Protein extracts were diluted to a concentration of 400 ng/μL using the rehydration/sample buffer (Bio Rad) and a total of 125 μL (50 μg protein) of this diluted protein solution was used to passively rehydrate linear, immobilized pH gradient (IPG) strips (BioRad; 7 cm, pH 4-7) overnight at room temperature. Isoelectric focusing of the proteins was performed on a PROTEAN IEF Cell (Bio Rad) and included a conditioning step (250 V for 15 min). The voltage was increased linearly to 4000 V over 2 h and focusing continued for an additional 5 h (20 000 VH). Focused IPG strips were equilibrated twice for 10 min each in 0.05 M Tris-HCl, pH 8.8, containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 2% (w/v) DTT, and then two times for 10 min each in the same buffer containing 2.5% (w/v) iodoacetamide instead of the DTT. Second dimension electrophoresis was performed using a Mini-PROTEAN 3 system (Bio Rad) for 80 minutes at 150 V. Following electrophoresis, gels were stained using the Silver Stain Plus kit (Bio Rad) according to the manufacturer's instructions.

Silver-stained gels were scanned using the GS-800 Calibrated Densitometer (Bio Rad) and images of the gels were compared using the PDQuest 2D analysis software (Bio Rad). The expected region of the gel where PR 10.1 protein normally migrates was carefully analyzed. The unique spot present in the transgenic plant in that region was excised using a sterile scalpel and placed in a microtiter plate containing 40 µL Milli Q water. The excised gel spot was prepared for mass spectrometry using a MassPREP Station (Micromass, Manchester, UK). LC/MS/MS analysis of the trypsin digested protein was performed using a PicoFrit capillary reversed-phase column (New Objectives, MA, USA) connected to a Quadrupole time of flight 2 mass spectrometer (Micromass, Manchester, UK.) The tandem mass spectrum (MS/MS) data was used to establish the identity of the excised protein.

3.3. Results and discussion

The transgenic *B. napus* (GN1-5#22) used in this study was one among several lines generated earlier in order to evaluate their resistance to diseases (Wang *et al.*, 1999). This particular transgenic line was chosen for our studies because of the relatively higher levels of PR 10.1 mRNA that was detected (Wang *et al.*, 1999) and seeds from T_2 plants were used in all experiments. In order to confirm the expression of the pea *PR 10.1* gene, we synthesized cDNA from RNA isolated from both roots and shoots of transgenic, as well as non-transgenic, wild-type seedlings. PCR analysis of the cDNA using *PR 10.1*-specific primers revealed the presence of an amplified product of the expected size (314 bp) in both roots (Figure 3-1A) and shoots (Figure 3-1B) of the transgenic seedlings. However, in the non-transgenic seedlings, a similar amplified product was absent, although the presence of the internal control (*18S rRNA*) was detected in all cases. The fact that an amplification product is not observed by RT-PCR in the non-transgenic *Brassica napus* germinated in 0 or 75 mM NaCl may be due to the pea *PR 10.1*-specific primers used in this study.

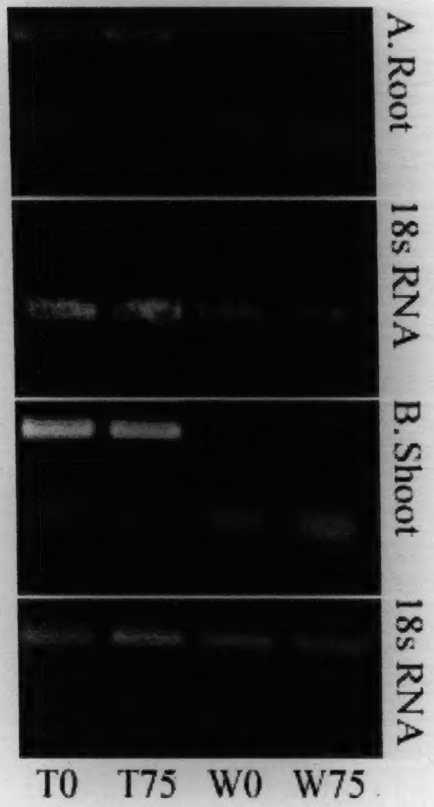

A. Root
18s RNA
B. Shoot
18s RNA
T0
T75
W0
W75

Figure 3-1. RT-PCR analysis of the expression of pea *PR 10.1* and *18S rRNA* genes in roots (A) and shoots (B) of *Brassica napus* L. seedlings. Seeds from wild-type (W) and transgenic (T) *B. napus* (cv. Westar) plants were germinated in 0 or 75 mM NaCl. Shoot and root tissue were excised from 7-day-old seedlings, RNA isolated and used for RT-PCR analysis using pea *PR 10.1*-specific primers.

Under the conditions used in these RT-PCR experiments, our primers specifically amplify only the pea *PR 10.1* gene as evidenced by the lack of amplification of the very homologous *PR 10.2* gene when a *PR 10.2* cDNA clone was used as a template (Tewari *et al.*, 2003^{b}).

The detection of mRNA for the transgene does not provide any evidence for the presence of the protein. Ideally, one can detect the presence of the protein by a Western blot, however the lack of antibodies to the pea PR 10 proteins precluded this. Therefore, to verify the presence of PR 10.1 protein in the transgenic seedlings we performed two-dimensional electrophoresis of protein extracted from the shoots of both control and transgenic seedlings. Silver stained images of the two-dimensional gels of protein extracts from the two seedlings are shown in Figures 3-2A and B.

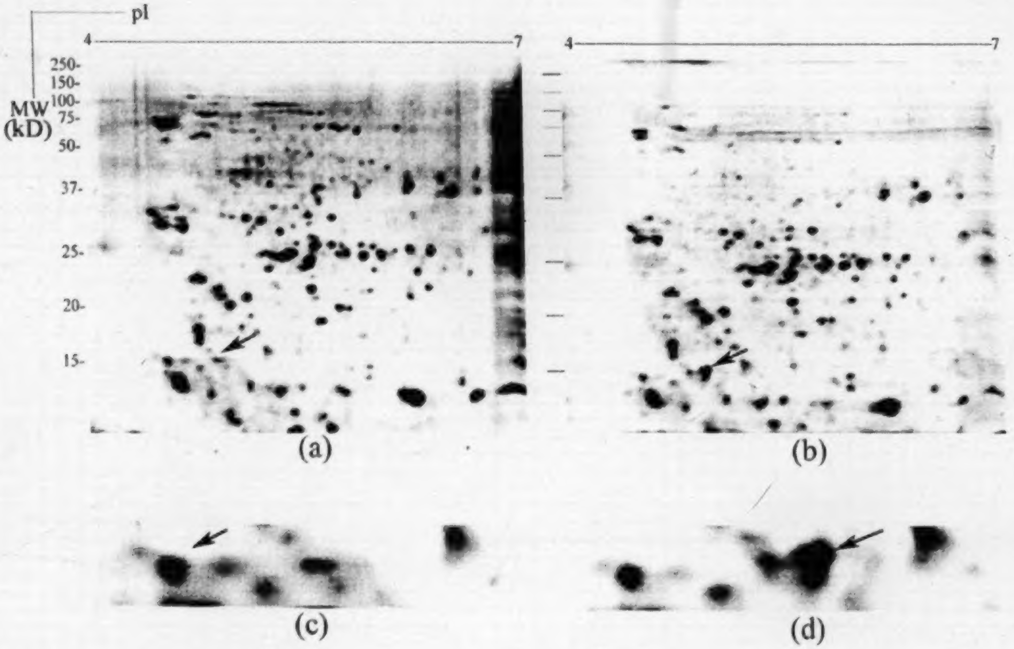


Figure 3-2. Two-dimensional electrophoresis of protein extracts prepared from the shoots of wild-type (A) and transgenic (B) *Brassica napus* L. seedlings. Wild-type and transgenic seeds were germinated for 7 days in water and total protein extracts prepared from isolated shot tissue. Protein extracts were subjected to two-dimensional electrophoresis and the protein spots visualized by silver staining. Panels (C) and (D) are subregions of the gels where a unique protein with the expected molecular weight and pI of pea PR 10.1 protein was detected. The location of this protein on all panels is indicated by the arrow.

The region of the gel where the pea PR 10.1 protein is expected to migrate is shown in Figures 3-2C and D from which it was clear that an unique protein was present in the transgenic shoot extracts. Again, due to the unavailability of specific antibodies, we decided to identify the unique protein via MS/MS. The MS/MS data was used to deduce the amino acid sequence of the peptides, which revealed the identity of the unique protein as pea PR 10.1 (Table 3-1). Our two-dimensional electrophoresis and mass spectrometry thus confirms the expression of the *PR 10.1* gene and the presence of the protein in transgenic *B. napus*.

Table 3-1. Disease resistance response protein Pi49 (PR 10) identified by ESI-Q-TOF

MS/MS (ESI-Q-TOF)		**Identity**	**[b]Access. No.**	**Mr /pI**
[a]PM/%	**Sequence**			
4/28%	LSAGPNGGSIAK LTFVEDGETK GDAAPSEEQLK ALVTDADNLTPK	Disease resistance response protein Pi49 (PR 10)	Gi|118933	16794/ 4.94

Note: [a] Number of peptides matched/ sequence percentage coverage.

[b] Accession number is Mascot search result using NCBI and other databases.

In order to assess the relative levels of salinity tolerance, particularly during germination, we germinated both control and transgenic seeds in Petri dishes with or without 75 mM NaCl. The appearance of the transgenic and non-transgenic seedlings after 7 days of continuous germination in Petri dishes in the absence or presence of 75 mM NaCl is shown in Figure 3-3. The appearance of five seedlings in a Petri dish that formed a typical experimental setup are shown in Figure 3-3A and representative control and transgenic seedlings in the absence or presence of NaCl are shown in Figure 3-3B. It is obvious that both the root and shoot lengths of the wild type seedlings were affected to a greater extent by the NaCl than transgenic seedlings. In order to fully gauge the extent of tolerance of transgenic seedlings to the imposed stress, their root and shoot lengths were measured and compared to the non-transgenic control. From the data presented (Figure 3-3C), it is evident that the mean shoot length of 7-day-old transgenic seedlings was significantly ($P<0.05$) greater than the wild-type controls. Furthermore, the extent of reduction of shoot length as a result of the salinity stress was lower in the case of transgenic seedlings, whereas in the control seedlings this reduction was significantly greater (Figure 3-3C). In the case of mean root lengths there were no significant differences between the transgenic and control seedlings in the absence of NaCl (Figure 3-3D). However, in the presence of NaCl, the extent of reduction in mean root length was once again significantly ($P< 0.05$) greater in the case of the non-transgenic, wild-type control (Figure 3-3D). There were no significant differences observed in the shoot masses of 7-day-old transgenic and control seedlings and root masses were too low, especially in the case of salinity stressed control seedlings, in order to make valid comparisons (data not shown).

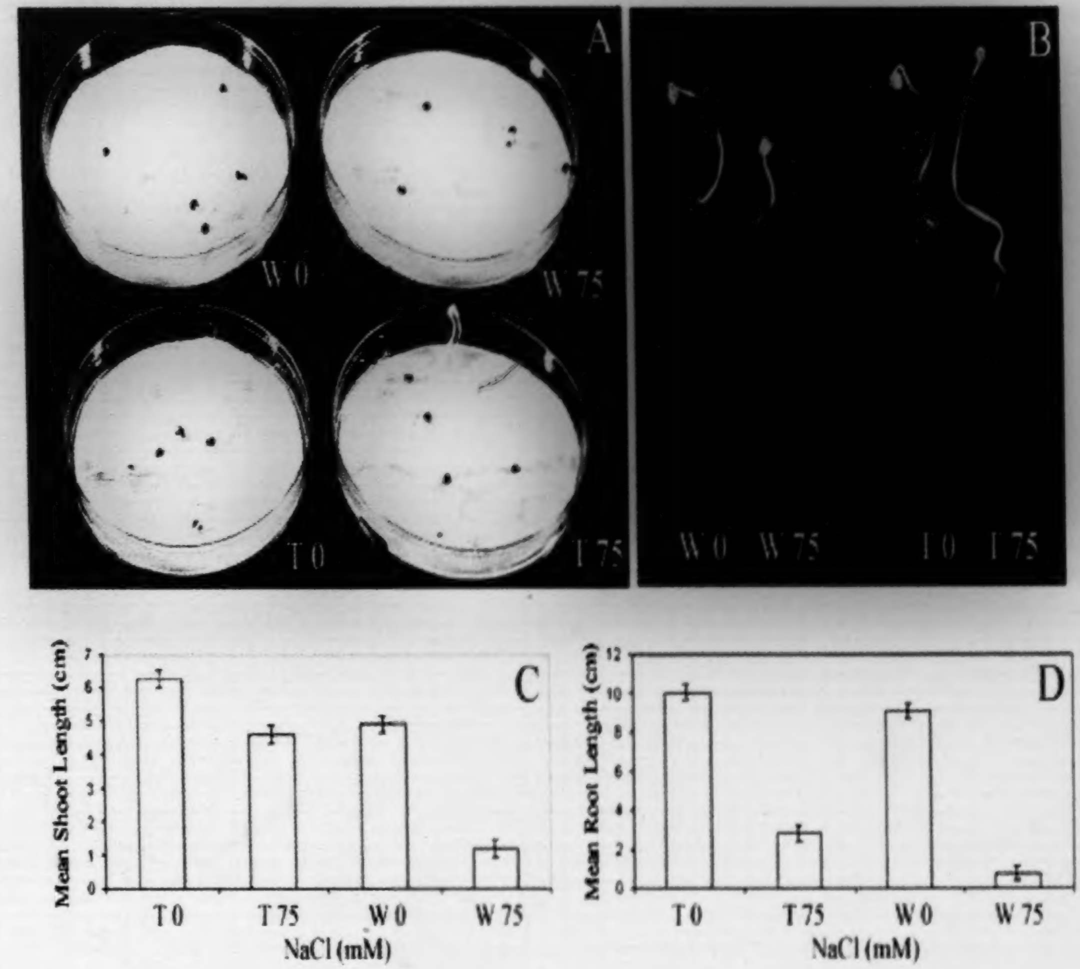


Figure 3-3. Effect of NaCl on the germination and subsequent growth of wild-type (W) and transgenic (T) *B. napus* seedlings. (A) Appearance of representative seedlings germinated in Petri dishes, (B) appearance of all seedlings in a typical Petri dish, (C) effect of NaCl on mean shoot length and (D) on the mean root length. An estimate of least square means and pooled SE are presented. Wild-type and transgenic seeds were germinated in water (0) or 75 mM NaCl (75), seedlings were photographed and the root and shoot lengths of 7-day-old seedlings were measured.

It is evident from the results presented that the pea *PR 10.1* gene is expressed and the protein product can be detected in the transgenic *B. napus* line. Furthermore, it is also evident that this transgenic line exhibits enhanced tolerance to salinity specifically during germination and subsequent seedling development, both of which are vital for improved crop productivity in marginal environments. Understandably, there is a need to investigate the mechanism by which PR 10.1 protein enhances the germination of *B. napus* in the presence of NaCl, as well as to test other members of the PR 10 protein family for their role(s) in mediating salinity (and perhaps other abiotic) stresses. Based on the preponderance of acidic amino acids in the primary structures of PR 10 proteins, Pnueli *et al.* (2002) proposed a LEA/dehydrin-like role for PR 10 proteins in the protection of cells from water-deficit stress damage. It would also be of interest to determine whether PR 10 proteins can ameliorate other water-deficit stresses such as drought and cold. Indeed, the observation that several members of western white pine PR 10 protein family are induced following cold-hardening, suggests that these proteins afford protection to a diverse range of stresses (Liu *et al.*, 2003).

Our findings demonstrated for the first time that a PR 10 protein is able to protect seedlings from salinity, which may have significance in the genetic engineering of water-deficit stress tolerance in plants. For example, it may be possible to use one or more members of the PR 10 family of proteins to further enhance the degree of tolerance to these stresses. Studies are currently underway in our laboratory to determine whether other members of the pea PR 10 protein family (specifically PR 10.2 and PR 10.4) enhance germination of canola under saline conditions. Moreover, the mechanism underlying PR 10-mediated salinity tolerance remains a conjecture at this point and should be investigated. We are in the process of overexpressing recombinant pea PR 10 proteins in *E. coli* for detailed structure and function analysis, which will provide insights into this aspect of PR 10 biology. In addition, we are in the process of characterizing the phytohormone profiles of the transgenic plants in order to explore the relationship between various hormones (especially cytokinins) and PR 10. Our studies and those of others will contribute to an enhanced understanding of the biology of PR 10 proteins for which a crucial role in plant stress tolerance has now been demonstrated.

3.4. References

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science **285**: 1256–1258.

Bantignies, B., Seguin, J., Muzac, I., Dedaldechamp, F., Gulick, P. and Ibrahim R. (2000) Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. Plant Mol Biol **42**: 871–881.

Biesiadka, J., Bujacz, G., Sikorski, M..M. and Jaskolski, M. (2002) Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. J Mol Biol **319**: 1223–1234.

Chang, M.M., Chiang, C.C., Martin, W.M. and Hadwiger, L.A. (1993) Expression of a pea disease resistance response gene in the potato cultivar Shepody Am Potato J **70**: 635–647.

Dubos, C. and Plomion C. (2001) Drought differentially affects expression of a PR-10 protein, in needles of maritime pine (*Pinus pinaster* Ait.) seedlings. J Exp Bot **52**: 1143–1144.

Duval, F.D., Renard, M., Jaquinod, M., Biou, V., Montrichard, F and Macherel, D. (2002) Differential expression and functional analysis of three calmodulin isoforms in germinating pea (*Pisum sativum* L.) seeds. Plant J **32**: 481–493.

Hasegawa, P.M., Bressan, R.A., Zhu, J-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51**: 463–499.

Kav, N.N.V., Srivastava, S., Goonewardene, L. and Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity. Ann Appl Biol **145:** 217–230.

Liu, J.-J., Ekramoddoullah, A.K.M. and Yu, X. (2003) Differential expression of multiple PR 10 proteins in western white pine following wounding, fungal infection and cold-hardening. Physiol Plant **119**: 544–553.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. and Yakovlev, G.I. (1994) High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193**: 470–472.

Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.S., Kim, D.H., Nam, J., Bahk, J., Hong, J.C., Lee, S.Y., Cho, M.J., Lim, C.O. and Yun, D.J. (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proc Natl Acad Sci U S A **100**: 358–363.

Park, C-J., Kim, K-J., Shin, R., Park, J. M., Shin, Y-C. and Paek, K-H. (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J **37**: 186–198.

Pnueli, L., Hallak-Herr, E., Rozenberg, M., Cohen, M., Goloubinoff, P., Kaplan, A. and Mittler, R. (2002) Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume *Retama raetam*. Plant J **31**: 319–330.

Riggleman, R.C., Fristensky, B. and Hadwiger, L.A. (1985) The disease resistance response in pea is associated with increased mRNAs. Plant Mol Biol **4**: 81–86.

Tewari, S., Brown, S.M. and Fristensky, B. (2003[a]) http://arXiv.org/q-bio.PE/0310003.

Tewari, S., Brown, S.M., Kenyon, P., Balcerzak, M., and Fristensky, B. (2003[b]) http://arXiv.org/q-bio.PE/0310038.

Wang, Y., Nowak, G., Culley, D., Hadwiger, L.A. and Fristensky, B. (1999) Constitutive expression of pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). Mol Plant-Microbe Interact **12**: 410–418.

Zhu, J-K. (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53**: 247-273.

4. Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin concentration

4.1. Introduction

Plant pathogenesis-related (PR) proteins are, as the name suggests, expressed in response to pathogen challenge, as well as during exposure of plants to abiotic stresses. They can be divided into at least 14 different families on the basis of their biological activity and are mostly secreted proteins (van Loon *et al.*, 1994). A notable exception to this rule are the proteins belonging to the class 10 family of PR proteins, the PR 10 protein family (van Loon *et al.*, 1994). PR 10 proteins are small (15-18 kDa), acidic, active in the cytosol and possess similar three-dimensional structures even though there may be significant differences at the level of their primary structures (van Loon *et al.*, 1994; Markovic-Housely *et al.*, 2003). Even though PR 10 proteins have been detected in a number of plant species and are known to be induced by pathogens or abiotic stresses, their precise biological activities are currently unknown.

Based on similarities in primary structures between plant PR 10 proteins and a ribonuclease from ginseng, it has been suggested that these proteins may be RNases that are involved in plant defense (Moiseyev *et al.*, 1994). This proposal has had some support due to the demonstrated RNase activity of some PR 10 proteins (Bantignies *et al.*, 2000; Wu *et al.*, 2003).

A version of this chapter has been published: ***Srivastava S.****, Emery R.J.N., Kurepin L.V., Reid D.M., Fristensky B. and Kav N.N.V. (2006) Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels.* ***Plant Growth Regulation,*** *49, 17–25.*

However, the proposed function of PR 10 proteins as RNases involved in plant defense has been questioned due to the expression of *PR 10* genes during normal growth and development of plants (Liu *et al*., 2003 and references therein). Moreover, researchers have also shown that PR 10 proteins bind many types of molecules including cytokinins, brassinosteroids, fatty acids and flavonoids (Fujimoto *et al*., 1998; Mogensen *et al*., 2002; Markovic-Housely *et al*., 2003) suggesting that all PR 10 proteins may not be RNases involved in plant defense. Thus, despite an increasing number of studies aimed at characterizing the role(s) of PR 10 proteins in plant processes during normal growth and development as well as during pathogen challenge, our understanding of their biological activities is far from being complete.

Abiotic stresses including salinity can also induce the expression of *PR 10* genes, suggesting a possible role in ameliorating the deleterious effects of stress (Ekramoddoullah *et al*., 1998; Hashimoto *et al*., 2004; Kav *et al*., 2004). Such a role is further supported by our observation that the constitutive expression of a pea *PR 10* gene in *B. napus* enhances their germination and early seedling growth under saline conditions (Srivastava *et al*., 2004). However, the precise biochemical activities of pea PR 10 proteins, as well as the mechanism(s) by which the pea PR 10 gene was able to enhance germination of *B. napus* are currently unknown. In this article, we overexpressed pea *PR 10.1* cDNA in *Escherichia coli*, purified the recombinant protein, and demonstrated that this protein possesses RNase activity. In order to further characterize the differences between the transgenic and wild type *B. napus* seedlings, we also determined the endogenous concentration of several phytohormones in both seedlings. The potential role(s) of pea PR 10 proteins with

RNase activity and the observed changes in phytohormone concentration in mediating plant responses to abiotic stress are discussed.

4.2. Materials and methods

4.2.1. Overexpression and purification of pea *PR 10.1* in *E. coli*

PR 10.1 cDNA was amplified using two specific primers; 5'-GTG GTC GCA TAT G**GA AAA TTT GTA CTT TCA AGG** TAT GGG TGT TTT TAA TGT TGA AGA TGA AAT CAC TTC TG-3' with a Nde I (underlined) and rTEV protease (bold) sites and 5'-TAT ATA GCT CGA GTT AGT TGT AAT CAG GAT GAG CCA AAC AGT AAC C-3' with a Xho I site (underlined). Amplified products were digested with NdeI and XhoI (New England Biolabs, ON, Canada) and cloned into pET28a bacterial expression vector (Novagen, WI, USA) for the expression of an N-terminal poly-histidine-tagged fusion protein and used to transform *E. coli* Rosetta (DE3) expression cells (Novagen). PR 10.1 protein was induced as recommended by the manufacturer (Novagen) using 1 mM IPTG, at RT for 3 h. Bacterial cells were harvested by centrifugation at 8300 g for 10 min at 4°C and the pellet resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, 1% Triton X100, complete EDTA-free protease inhibitor cocktail (Roche Inc., USA), pH 7.5].

Cells were lysed using a SONIC 300 Dismembrator (Artek Systems Corp., NY, USA) with 10 cycles of 30 sec each at a setting of 0.8 relative output with one minute cooling step (on ice) between each cycle. After centrifugation (13,800 g, 10 min, 4ºC), the supernatant was applied to a Ni-NTA agarose (Qiagen) column equilibrated with lysis buffer. The column was washed with 20 column volumes of wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM β-mercaptoethanol, 1% Triton X100, 10% glycerol, 15 mM imidazole, complete EDTA-free protease inhibitor cocktail, pH 7.5) and then with 15 column volumes of wash buffer 2 (same as buffer 1 but with 30 mM imidazole) to remove weakly bound bacterial proteins. The PR 10.1 protein was eluted from the column with wash buffer 3 (same as buffer 1 with 150 mM imidazole). The eluted, histidine-tagged protein was dialyzed overnight against rTEV protease buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT) at 4ºC. The histidine tag was removed by incubation with 1.2 U/µg of the rTEV protease (Invitrogen, CA, USA) for 5 h at 30ºC followed by an overnight incubation at 4ºC and subsequent chromatography on Ni-NTA agarose column and collection of the flow through.

4.2.2. RNase activity measurements

In-solution RNase activity assays with the purified, recombinant PR 10.1 were performed as described by Bantignies *et al*. (2000) with modifications. Reaction mixtures containing 6 µg total RNA from either pea or canola tissue and 3, 6 or 9 µg uncleaved or cleaved proteins in rTEV buffer (see above) were incubated at RT for 3 h, extracted with equal volume of phenol-chloroform and the aqueous layer was analyzed on a 1.2% agarose gel. RNA was isolated from the roots of 7-day-old pea seedlings or from the shoots of 2-week-old canola plants as described earlier (Srivastava *et al.*, 2004).

In-gel RNase activity assays with recombinant pea PR 10.1 as well as with crude, pea seedling root extracts were performed as described by Yen and Green (1991). The glass plates were treated with 0.1% DEPC overnight and autoclaved. The gel casting units were treated with RNase Zap (Ambion Inc, USA). The 15% acrylamide gel was cast with 2.4 mg/mL yeast tRNA and recombinant or crude protein extract was mixed with gel loading dye and loaded in the well without boiling. The protein separation was performed in a Mini PROTEAN 3 vertical slab system (Bio-Rad) at constant voltage (160 V) until the dye front reached the bottom of the gel. After electrophoresis the gels were washed twice with 25% isopropanol (v/v) in 0.01 M Tris-HCl for 10 min each at RT (20±2°C) to remove SDS and renature the protein. The gels were subsequently washed twice with 0.01 M Tris-HCl for 10 min each at RT (20±2°C) to remove the isopropanol. Following the washing steps, gels were incubated in 0.1 M Tris-HCl at 51°C for 50 min to allow the RNases to renature and digest the tRNA in the gel. Gels were stained with 0.2% (w/v) toluidine blue O (Aldrich Inc., USA) in 0.01 M Tris-HCl for 10 min at RT and destained by incubating twice with 0.01 M Tris-HCl for 10 min each after which the gels were rinsed and stored in 10 % glycerol-0.01 M Tris-HCl.

4.2.3. Phytohormones analysis

Transformation of canola (*Brassica napus* L. cv. Westar) with the pea *PR 10.1* cDNA and its constitutive expression in the transgenic line GN1-5#22 has been previously described (Wang *et al*., 1999; Srivastava *et al*., 2004). The experiment was designed in a randomized complete block design and wild type and transgenic seeds (from T_4 plants) were germinated and grown for 7 days in the dark at room temperature (20±2°C). The plates were sealed with laboratory film and after 7 days the entire seedlings were freeze-dried for phytohormone analysis. Fresh 7-day-old *B. napus* (transgenic and Westar) tissue was collected and immediately frozen in liquid N_2 and later freeze-dried. The freeze-dried tissue was ground in liquid nitrogen and extracted with 80% methanol. $[^2H_6]$ ABA (250 ng), $[^{13}C_6]$ IAA (200 ng) and 20 to 33 ng each of $[^2H_2]$ GA_1 and $[^2H_2]$ GA_{20} were added to the aqueous MeOH extracts as internal standards. The extract was then subjected to reversed phase C_{18} HPLC separation followed by identification and quantification analysis on a gas chromatograph connected to a mass spectrometer (GC-MS; Agilent 6890) using the -selected ion monitoring (-SIM) mode, as described in Fellner *et al*. (2001). Quantification was accomplished by reference to the stable isotope-labeled internal standard using equations for isotope dilution analysis, adapted by DW Pearce (see Jacobsen *et al*., 2002) from Gaskin and MacMillan (1991). For CK analysis, 7-day-old *B. napus* seedlings were extracted, purified and quantified by LC-(+) ESI-MS/MS using the isotope dilution method as described in Ferguson *et al*. (2005). One hundred nanograms of the following were added as internal standards: $[^2H_6]$iP, $[^2H_6]$[9R]iP, *trans*-$[^2H_5]$Z, $[_2H_3]$DZ, *trans*-$[^2H_5]$[9R]Z, $[^2H_3]$[9R]DHZ, $[^2H_6]$[9RMP]iP, *trans*-$[^2H_5]$[9RMP]Z and $[^2H_3]$[9RMP]DHZ (OlChemIm Ltd, Olomouc, Czech Republic).

4.3. Results and discussion

The recombinant protein was purified from the soluble fraction by chromatography on Ni-NTA column (Figure 4-1). RNase activities of recombinant pea PR 10.1, with or without the histidine tag, were determined using an in-solution, as well as an in-gel RNA degradation assay (Figure 4-2). It was evident that both forms of the protein possessed RNase activity capable of hydrolyzing pea and canola RNA (Figure 4-2A and B). In order to confirm that the PR 10.1 protein is a RNase, we performed in-gel RNase assays as described by Yen and Green (1991) and visualized by staining with toluidine blue O (Yen and Green, 1991). Arrows point to the clear bands that are indicative of RNase activity.

These in-gel assays produce gels with a dark background and proteins with RNase activities are detected on the basis of the clear region observed at molecular weights corresponding to the protein(s) of interest. Results shown in Figure 2C clearly demonstrate that both forms of recombinant PR 10.1 protein (with or without the histidine tags) possess RNase activity. Also included in Figure 4-2C is a crude pea seedling root protein extract where RNase activity is observed at the molecular weight (~16 kDa) corresponding to PR 10 proteins indicating that the native proteins also possess RNase activity.

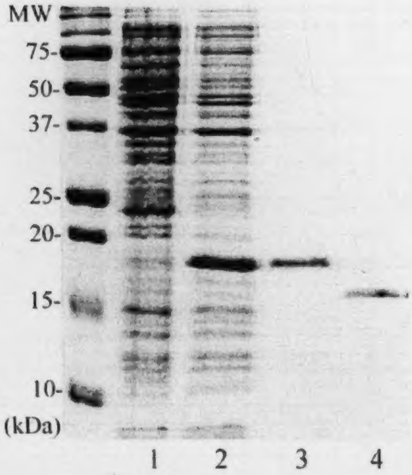


Figure 4-1. Over expression and purification of pea (*P. sativum*) PR 10.1. Lanes 1, 2 are cell-free extracts from uninduced and induced *E. coli* cultures, respectively and lanes 3 and 4 are the purified, recombinant PR 10.1 proteins with or without the N-terminal poly-histidine tag, respectively.

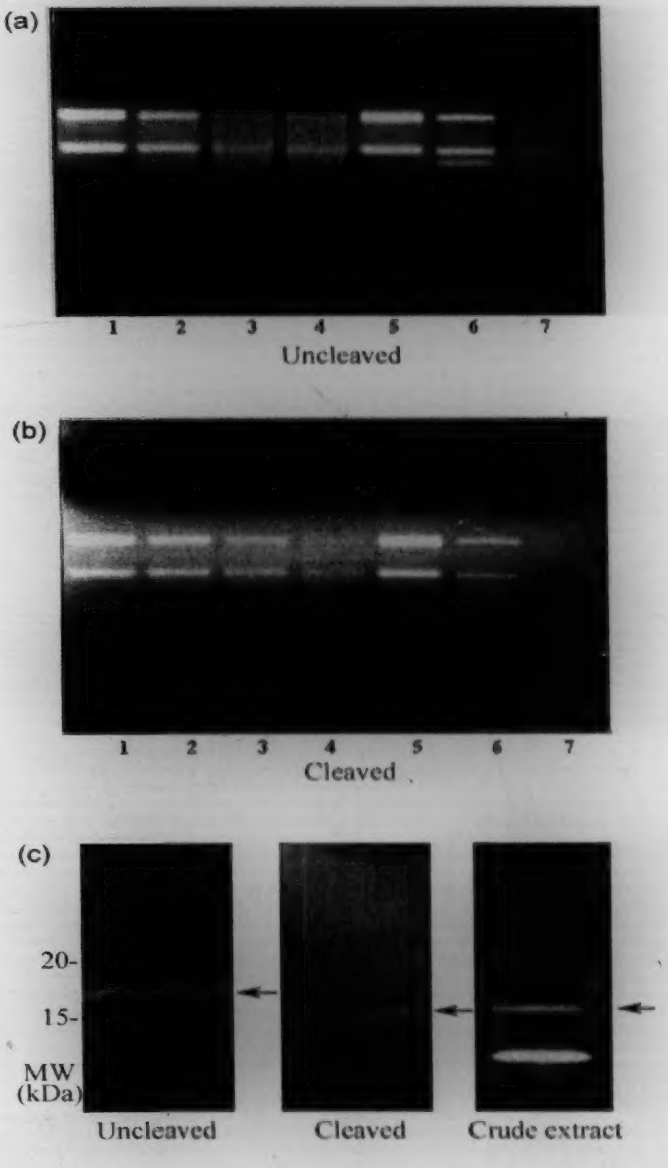

(a)
1 2 3 4 5 6 7
Uncleaved
(b)
1 2 3 4 5 6 7
Cleaved
(c)
20-
15-
MW (kDa)
Uncleaved
Cleaved
Crude extract

Figure 4-2. Ribonuclease activity of pea (*Pisum sativum*) PR 10 protein. In-solution RNA degradation assay with histidine-tagged recombinant PR 10.1 (a) or recombinant PR 10.1 without the histidine tag (b). In both panels A and B, lanes 1-4 are reactions with total RNA from pea and 0, 3, 6 or 9 μg recombinant PR 10.1 protein; lanes 6 and

7 with total RNA from canola and 0 or 9 μg recombinant PR 10.1 protein. Lane 5 is total RNA from pea incubated with recombinant PR 10.1 protein (9 μg) which had been boiled in a boiling water bath for 15 min. In-gel RNA degradation assay (c); from left to right are purified, recombinant histidine-tagged protein, cleaved recombinant protein and crude pea seedling root extract. SDS-PAGE gels containing yeast tRNA are used for electrophoresis and protein bands with RNase activity are visualized by staining with toluidine blue O (Yen and Green 1991). Arrows point to the clear bands which are indicative of RNase activity.

As described earlier, PR 10 proteins are induced by abiotic, as well as biotic stresses. For example, infection of pea with *Fusarium solani* (Fristensky *et al.*, 1985), rice with *Magnaporthe grisea* (McGee *et al.*, 2001), barley with *Rhynchosporium secalis* (Steiner-Lange *et al.*, 2003), alfalfa with *Cuscuta trifolii* (Borsics and Lados 2002) and sorghum with *Cochliobolus heterostrophus* (Lo *et al.*, 1999) has been reported to induce PR 10 proteins. The ribonuclease activity of PR 10 proteins suggests a potential role in defense against pathogenic infections and it has been demonstrated that the constitutive expression of a pea *PR 10* gene in potato confers resistance to potato early dying disease (Chang *et al.*, 1993). However, no resistance to the fungal pathogen *Leptosphaeria maculans* was observed in transgenic *B. napus* constitutively expressing the pea *PR 10.1* gene (Wang *et al.*, 1999). Recently, it was demonstrated that a capsicum PR 10 protein has RNase-mediated antiviral activity against Tobacco Mosaic Virus (TMV) which was enhanced by phosphorylation (Park *et al.*, 2004). Although numerous articles report the elevation of PR 10 proteins by various abiotic stresses, our previous finding that the constitutive expression of a pea PR 10 protein enhances germination under saline conditions remains the only report that PR 10 confers a positive effect on growth (Srivastava *et al.*, 2004).

The constitutive expression of the pea *PR 10.1* cDNA in *B. napus* minimizes the deleterious effects of salinity on root and shoot growth (Srivastava *et al.*, 2004). In the absence of NaCl, there were no appreciable differences in the mean root lengths of the 7-day-old transgenic seedlings compared to wild type seedlings of a similar age, whereas the mean shoot lengths were slightly higher (Srivastava *et al.*, 2004). The appearance of the wild type and transgenic seedlings of different chronological ages is shown in Figure 4-3. It is apparent that the radicles of the transgenic seedlings are better developed at 48 h than those of the wild type. This may have resulted from the earlier (~12 h) germination of the transgenic seeds; although, both transgenic and wild type seeds had completed germination within the first 24 h (Figure 4-3A). In the case of 7-day-old transgenic and wild type seedlings (6 days after germination) grown either in Petri dishes or soil, the observed differences were negligible supporting our previous observations that phenotypic differences of 7-day-old seedlings are minimal (Figures 4-3B, C).

In order to further characterize the differences between the transgenic and wild type *B. napus* seedlings that may help in understanding the basis for the observed tolerance to salinity during early seedling growth (Srivastava *et al.*, 2004), we investigated whether the changes were hormonally mediated. We determined the endogenous concentrations of four phytohormones groups in 7-day-old seedlings (Table 4-1). Seven-day-old seedlings were used for this investigation in order to obtain sufficient tissue to perform all the phytohormone analyses, as well as to ensure that there would be minimal developmental differences. No significant differences in IAA, GA_1 and GA_4 concentrations were observed, and GA_9 could not be detected.

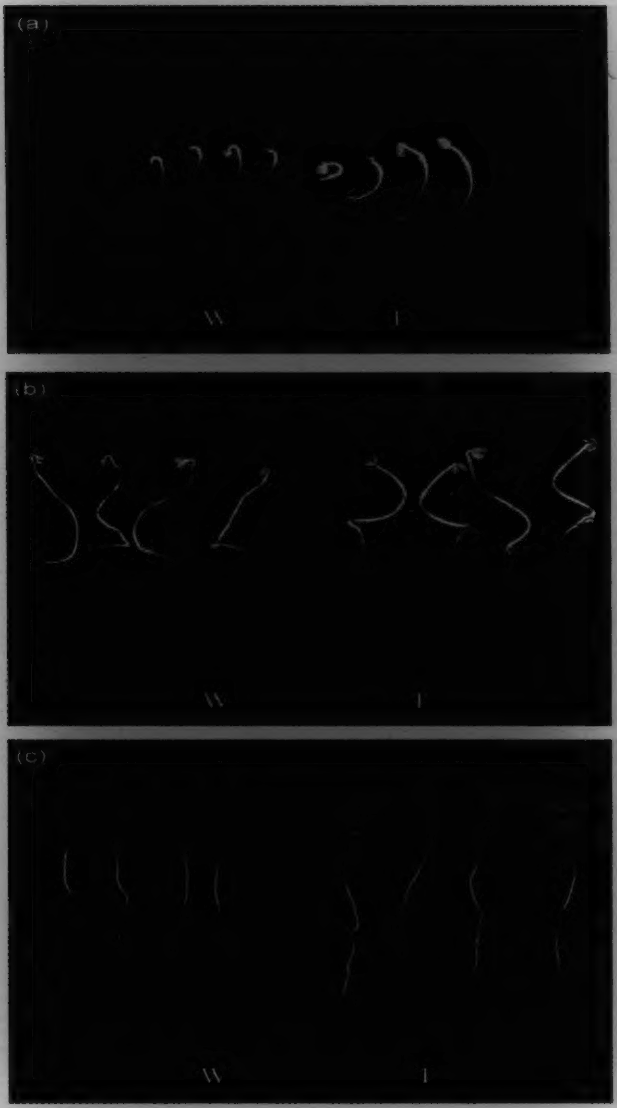


Figure 4-3. Comparison of germination and early seedling growth of 7-day-old wild type and transgenic (pea *PR 10.1*) *B. napus* seedlings. Appearance of wild type (W) and PR 10.1 transgenic (T) seedlings after 2 days (a) and 7 days (b) after germination and grown in Petri dishes. (c) Appearance of 7-day-old wild type (W) and transgenic (T) seedlings in soil.

Table 4-1. Endogenous concentration of various hormones in 7-day-old wild type and transgenic (pea *PR 10.1*) *B. napus* seedlings

Hormone	**Concentration** **Ng/g DW^{-1} (n = 2)**	
	Wild-type (Mean ± SE)	**Transgenic** (Mean ± SE)
GA1	0.57 ± <0.01	0.48 ± 0
GA4	5.5 ± 0.9	5 ± 0.3
GA20	10.2 ± 1.1	19.2 ± 0.7
IAA	130 ± 7	119 ± 8
ABA	217 ± 4	106 ± 19
Cytokinins	Pmol gDW^{-1} (n = 2)	
***Trans*-Z**	118 ± 0.2	273 ± 67
***cis*-Z**	not detected	not detected
iP	2 ± 0.8	0.9 ± <0.1
***trans*-[9R]Z**	52 ± 18.4	4865 ± 2263
***cis*-[9R]Z**	32 ± 28	15 ± 5
[9R]DHZ	2.5 ± 0.9	58 ± 28
[9R]iP	2.7 ± 2.4	3.6 ± 0.7
***trans*-[9RMP]Z**	33 ± 5	1614 ± 701
***cis*-[9RMP]Z**	101 ± 19	101 ± 12
[9RMP]DHZ	10.3 ± 2.8	13.7 ± 4.9
[9R]iP	6.3 ± 0.3	6.3 ± 0.5
Cytokinin free Bases (FB)	210 ± 7	5215 ± 2352
Cytokinin Nucleotides (NT)	150 ± 26	1734 ± 718
Total cytokinins	360 ± 18	6949 ± 3071

The endogenous concentration of GA_{20} was higher in *PR 10.1* seedlings (Table 4-1); however, since GA_{20} is not considered to be biologically active (Santes and Garcia-Martinez, 1995), it is unlikely that this difference would have contributed to the increased salinity tolerance of the transgenic seedlings. However, the endogenous concentration of ABA reduced 50% and cytokinin concentration increased extensively in the transgenic seedlings (Table 4-1).

Abscisic acid (ABA) regulates a number of physiological processes in plants including the onset and maintenance of dormancy (Finkelstein *et al.*, 2002, Gubler *et al.*, 2005). The relationship between ABA and GA during germination is well known. For example, ABA delays or prevents seed germination and determines dormancy levels whereas GA breaks dormancy and promotes germination (Groot and Karssen, 1992; Koornneef *et al.*, 2002). A relationship between CK and ABA has also been reported in the literature including that by Chang *et al.* (2003) where transgenic petunia flowers with elevated concentration of cytokinins as a result of isopentenyl transferase (*IPT*) overexpression significantly reduces the concentration of ABA. The observations of Chang *et al.* (2003) are consistent with our findings that the transgenic seedlings constitutively expressing the pea *PR 10.1* cDNA have a lower endogenous ABA concentration compared to the wild type seedlings (Table 4-1). This reduction of ABA combined with the observed increases in cytokinin (CK) concentration may contribute to the enhanced growth of the transgenic seedlings (see below).

A massive increase in the total cytokinin was observed in 7-day-old transgenic seedlings (Table 4-1). Changes in CK were comprised of the free base and ribosides (which are presumed to be active), as well as the nucleotide forms. More specifically, the biggest difference observed was a dramatic increase in the concentration of *trans*-zeatin riboside (*trans*-[9R]Z) and its nucleotide (*trans*-[9RMP]Z) in the transgenic seedlings. Much smaller increases were observed in the concentration of *trans*-zeatin and *trans*-[9RMP]DHZ in the transgenic seedlings (Table 4-1). No differences in the concentration of [9R]iP, iP or *cis*-[9R]Z were observed between the two types of seedlings, and *cis*-Z was not detected in either genotype (Table 4-1). Due to the large number (>1000) of seedlings required to perform the endogenous phytohormone analyses, the numbers in Table 4-1 reflect the average values from two independent extractions and analyses from seedlings that were pooled from several biological replicates.

Cytokinins are plant hormones that have been isolated from a number of species and are known to be synthesized by the embryonic axes of germinating seedlings (Villalobos and Martin, 1992). Cytokinins may also be involved in post-germination processes such as root and hypocotyl growth (Singh and Sawhney, 1992). Two *de novo* pathways for cytokinin biosynthesis have been demonstrated in plants (Kakimoto, 2003) and are thought to be the major sources for endogenous, free cytokinins. The significance of such a large increase in CK is that they are thought to play an important role in mediating plant responses to stresses (Harding and Smigocki, 1994; Gan and Amasino, 1995). For example, it has been demonstrated that CKs may function as antioxidants in germinating soybean seedlings (Gidrol *et al.*, 1994); synthetic cytokinin analogues possess antioxidant activities (Brathe *et al.*, 2002); and CKs induce a metallothionein gene during copper stress (Thomas *et al.*, 2005). The fact that an elevated CK concentration may contribute in enhancing growth of transgenic seedlings under saline conditions is supported by the fact that the greatest increases in the *PR 10.1* transgenic seedlings were in *trans*-[9R]Z, one of the most active CKs in all known bioassays, and another potentially active form, *trans*-[9RMP]Z (Emery and Atkins, 2005).

Combined with the stress-induced expression of plant PR 10 proteins, a possible implication is that PR 10 proteins mediate their functions through altering endogenous cytokinin concentration. Indeed, it is possible that the observed RNase activity of the PR 10 proteins may be responsible for the increase in cytokinin concentration since in addition to *de novo* biosynthetic pathways, cytokinins may also be derived from modified bases that occur in plant tRNAs (Skoog and Armstrong, 1970; Haberer and Kieber, 2002). Furthermore, tRNA has been demonstrated to be a source of cytokinin and it is possible that their turnover contributes to as much as 40-50% of the pool of free active cytokinins (Barnes *et al.*, 1980; Letham and Palni, 1983; Taller *et al.*, 1987; Prinsen *et al.*, 1997). However, the isomer profiles of CK detected in this study are not consistent with this hypothesis. Since the major CK moiety present in plant tRNAs is *cis*-Z (Taller, 1994), the degradation of tRNA by PR 10.1 would be expected to enhance the concentration of *cis*-Z, *cis*-[9R]Z and *cis*-[9RMP]Z. Our results show (Table 4-1), that there were no difference in the concentration of *cis*-[9RMP]Z, and a reduction in the concentration of *cis*-[9R]Z and *cis*-Z could not be detected. The possibility remains that that both *cis*-[9R]Z, as well as *cis*-Z, were converted to their respective *trans* forms by a *cis-trans* isomerase, like that isolated from *Phaseolus vulgaris* endosperms (Bassil *et al.*, 1993; Mok and Mok, 2001). Moreover, *trans*-Z has been detected in the tRNAs of hop plants (*Humulus* *lupulus* L.) where it constitutes 25% of total tRNA zeatin (Wang, 1994). However, neither *P. vulgaris* nor hop plants belong to the same family as *B. napus* and the presence of an isomerase and/or *trans*-Z (or its nucleotide forms) in *B. napus* tRNA must be investigated. Another issue that warrants discussion at this stage is the fact that the concentration of the active *trans*-Z in the transgenic seedlings were only ~2-fold higher whereas the concentration of both *trans*-[9R]Z and *trans*-[9RMP]Z were at least 30-fold higher (Table 4-1). This may be due to the fact that *trans*-[9R]Z can be converted to either [9R]-DHZ or to *trans*-[9RMP]Z (Takei *et al*., 2004). It should be stressed that the endogenous CK concentrations were determined at a specific developmental stage (6 days after germination) that will undoubtedly have a significant effect on the proportion of individual species that are present.

In conclusion, although it is tempting to speculate that the RNase activity of pea PR 10 protein may lead to the enhanced endogenous cytokinin pool in the transgenic *B. napus*, it is equally possible that the PR 10 protein mediates the increase in cytokinins by some other mechanism, including increased *de novo* biosynthesis or reduced degradation/oxidation. Alternatively, the differences in endogenous CK concentration may be caused by subtle differences in seedling development, although both wild type and transgenic seedlings used for phytohormone analyses were at 6 days after germination. To rule out this possibility, such hormone analyses must be performed encompassing a window of several days during germination and early seedling growth. To unequivocally test the role of RNase in elevating the CK concentration in transgenic seedlings, we have initiated these as well as additional experiments that include the feeding of radiolabelled tRNA (to determine the incorporation of the radiolabel in individual species of cytokinins) and the detection of any active *cis-trans* isomerase in *B. napus*. We are also in the process of expressing other *PR 10* homologues in *Arabidopsis thaliana* in order to investigate the effects of *PR 10* on cytokinin biosynthesis, degradation, conjugation, and signal transduction in a system with more comprehensive genomic resources.

4.4. References

Bantignies, B., Seguin, J., Muzac, I., Dedaldechamp, F., Gulick, P. and Ibrahim, R. (2000) Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. Plant Mol Biol **42:** 871–881.

Barnes, M.F., Tien, Ch.L. and Gray, J.S. (1980) Biosynthesis of cytokinins by potato cell cultures. Phytochemistry **19:** 409–412.

Bassil, N.V., Mok, D.W.S. and Mok, M.C. (1993) Partial purification of a *cis-trans*-isomerase of zeatin from immature seeds of *Phaseolus vulgaris* L. Plant Physiol **102:** 867–872.

Borsics, T. and Lados, M. (2002) Dodder infection induces the expression of a pathogenesis-related gene of the family PR-10 in alfalfa. J Exp Bot **53:** 1831–1832.

Brathe, A., Andresen, G., Gundersen, L.L., Malterud, K.E. and Rise, F. (2002) Antioxidant activity of synthetic cytokinin analogues: 6-alkynyl- and 6-alkenylpurines as novel 15-Lipoxygenase inhibitors. Bioorg Med Chem **10:** 1581–1586.

Chang, M.M., Chiang, C.C., Martin, W.M. and Hadwiger, L.A. (1993) Expression of a pea disease resistance response gene in the potato cultivar Shepody. Amer Potato J **70:** 635–647.

Chang, H., Jones, M.L., Banowetz, G.M. and Clark, D.G. (2003) Overproduction of cytokinins in petunia flowers transformed with P(SAG12)-IPT delays corolla senescence and decreases sensitivity to ethylene. Plant Physiol **132:** 2174–2183.

Ekramoddoullah, A.K.M., Davidson, J.J. and Taylor, D. (1998) A protein associated with frost hardiness of western white pine is up-regulated by infection in the white pine blister rust pathosystem. Can J For Res **28:** 412–417.

Emery, R.J.N. and Atkins, C.A. (2005) Cytokinins in Seed Development. In: Amarjit S.B. (ed.), Handbook of Seed Science. Haworth's Food Products Press, Binghamton, New York (in press).

Fellner, M., Zhang, R., Pharis, R.P. and Sawhney, V.K. (2001) Reduced de-etiolation of hypocotyl growth in a tomato mutant is associated with hypersensitivity to, and high endogenous concentration of abscisic acid. J Exp Bot **52:** 725–738.

Ferguson, B.J., Wiebe, E.M., Emery, R.J.N. and Guinel, F.C. (2005) Cytokinin accumulation and an altered ethylene response mediate the pleiotropic phenotype of the pea nodulation mutant R50 (sym16). Can J Bot **83:** 989–1000.

Finkelstein, R.R., Gampala, S.S. and Rock, C.D. (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell **14:** S15-45.

Fristensky, B., Riggleman, R.C., Wagoner, W. and Hadwiger, L.A. (1985) Gene expression in susceptible and disease resistant interactions of pea induced with *Fusarium solani* pathogens and chitosan. Physiol Plant Pathol **27:** 15–28.

Fujimoto, Y., Nagata, R., Fukasawa, H., Yano, K., Azuma, M., Iida, A., Sugimoto, S., Shudo, K. and Hashimoto, Y. (1998) Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (*Vigna radiata*). Eur J Biochem **258:** 794–802.

Gan, S. and Amasino, R.M. (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. Science **270:** 1986–1988.

Gaskin, P. and MacMillian, J. (1991) GC-MS of the gibberellins and related compounds. In: Methodology and a library of spectra. University of Bristol (Cantock's Enterprises), Bristol, England, UK.

Gidrol, X., Lin, W., Degousee, N., Yip, S. and Kush, A. (1994) Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. Eur J Biochem **224:** 21–28.

Groot, S.P.C. and Karssen, C.M. (1992) Dormancy and germination of abscisic acid-deficient tomato seeds. Plant Physiol **99:** 952–958.

Gubler, F., Millar, A.A. and Jacobsen, J.V. (2005) Dormancy release, ABA and pre-harvest sprouting. Curr Opin Plant Biol **8:** 183–187.

Haberer, G. and Kieber, J.J. (2002) Cytokinins. New insights into a classic phytohormone. Plant Physiol **128:** 354–362.

Harding, S. and Smigocki, A. (1994) Cytokinins modulate stress response genes in isopentenyl transferase-transformed *Nicotiana plumbaginifolia* plants. Physiol Plant **90:** 327–333.

Hashimoto, M., Kisseleva, L., Sawa, S., Furukawa, T., Komatsu, S. and Koshiba, T. (2004) Novel rice PR 10 protein, RSOsPR 10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. Plant Cell Physiol **45:** 550–559.

Jacobsen, J.V., Pearce, D.W., Poole, A.T., Pharis, R.P. and Mander, L.N. (2002) Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. Plant Physiol **115:** 428–441.

Kakimoto, T. (2003) Biosynthesis of cytokinins. J Plant Res **116:** 233-239.

Kav, N.N.V., Srivastava, S., Goonewardene, L. and Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity. Ann Appl Biol **145:** 217-230.

Koornneef, M., Bentsink, L. and Hilhorst, H. (2002) Seed dormancy and germination. Curr Opin Plant Biol **5:** 33–36.

Letham, D.S. and Palni, L.M.S. (1983) The Biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol **34:** 163–197.

Liu, J.-J., Ekramoddoullah, A.K.M. and Yu, X. (2003) Differential expression of multiple PR 10 proteins in western white pine following wounding, fungal infection and cold-hardening. Physiol Plant **119:** 544–553.

Lo, S.C., Hipskind, J.D. and Nicholson, R.L. (1999) cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defense-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. Mol Plant Microbe Interact **12:** 479–489.

Markovic-Housely, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. and Breitender, H. (2003) Crystal structure of a hypoallergenic isoforms of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J Mol Biol **325:** 123–133.

McGee, J.D., Hamer, J.E. and Hodges, T.K. (2001). Characterization of a PR-10 pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. Mol Plant Microbe Interact **14:** 877–886.

Mogensen, J.E., Wimmer, R., Larsen, J.N. and Spangfort, M.D. (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J Biol Chem **277:** 684–692.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. and Yakovlev, G.I. (1994) High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193:** 470–472.

Mok, D.W. and Mok, M.C. (2001) Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol **52:** 89–118.

Park, C.J., Kim, K.J., Shin, R., Park, J.M., Shin, Y.C. and Paek, K.H. (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J **37:** 186–198.

Prinsen, E., Kaminek, M. and van Onckelen, H.A. (1997) Cytokinin biosynthesis: a black box? Plant Growth Reg **23:** 3–15.

Singh, S. and Sawhney, V.K. (1992) Endogenous hormones in seeds, germination behaviour and early seedling characteristics in a normal and ogura cytoplasmic male sterile line of rapeseed (*Brassica napus* L.). J Exp Bot **43:** 1497–1505.

Santes, C.M. and Garcia-Martinez, J. (1995) Effect of the growth retardant 3, 5-Dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester, an acylcyclohexanedione compound, on fruit growth and gibberellin content of pollinated and unpollinated ovaries in Pea. Plant Physiol **108:** 517–523.

Skoog, F. and Armstrong, D.J. (1970) Cytokinins. Annu Rev Plant Physiol **21:** 359–384.

Srivastava, S., Fristensky, B. and Kav, N.N.V. (2004) Constitutive expression of a PR 10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol **45:** 1320–1324.

Steiner-Lange, S., Fischer, A., Boettcher, A., Rouhara, I., Liedgens, H., Schmelzer, E. and Knogge, W. (2003) Differential defense reactions in leaf tissues of barley in response to infection by *Rhynchosporium secalis* and to treatment with a fungal avirulence gene product. Mol Plant Microbe Interact **16:** 893–902.

Takei, K., Yamaya, T. and Sakakibara, H. (2004) *Arabidopsis* CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin. J Biol Chem **279:** 41866–41872.

Taller, B.J., Murai, N. and Skoog, F. (1987) Endogenous cytokinins in the ribosomal RNA of higher plants. Plant Physiol. **83:** 755–760.

Taller, B.J. (1994) Distribution, biosynthesis and function of cytokinins in tRNA. In: Mok D.W.S. and Mok. M.C. (eds.), Cytokinins, Chemistry, Activity and Function, CRC Press, Boca Raton, USA, pp. 101–112.

Thomas, J.C., Perron, M., LaRosa, P.C. and Smigocki, A.C. (2005) Cytokinin and the regulation of a tobacco metallothionein-like gene during copper stress. Physiol Plant **123:** 262-271.

van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Report **12:** 245–264.

Villalobos, N. and Martin, L. (1992) Involvement of cytokinins in the germination of chick-pea seeds. Plant Growth Regul **11:** 277–291.

Wang, T.L. (1994) Cytokinin mutants. In: Mok D.W.S. and Mok M.C. (eds.), Cytokinins: Chemistry, Activity and Function, CRC Press, Boca Raton, USA, pp. 255–268.

Wang, Y., Nowak, G., Culley, D., Hadwiger, L.A. and Fristensky, B. (1999) Constitutive expression of pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). Mol. Plant-Microbe Interact **12:** 410–418.

Wu, F., Li, Y.K., Chang, S., Zhou, Z., Wang, F., Song, X., Lin, Y. and Gong, W. (2003) Purification, characterization and preliminary crystallographic studies of a PR-10 protein from *Pachyrrhizus erosus* seeds. Acta Crystal D **58:** 2165–2167.

Yen, Y. and Green, P.J. (1991) Identification and properties of the major ribonucleases of *Arabidopsis thaliana*. Plant Physiol **97:** 1487–1493.

5. Constitutive expression of the pea ABA-responsive 17 (*ABR17*) cDNA confers multiple stress tolerance in *Arabidopsis thaliana*

5.1. Introduction

Plants encounter a wide range of environmental stresses, including drought, salinity, temperature extremes, flooding and ultraviolet radiation, which severely limit crop productivity worldwide (Boyer, 1982). Abiotic stresses can occur simultaneously and could affect multiple stages of plant growth and development (Chinnusamy *et al.*, 2004), leading to serious morphological, physiological, biochemical and molecular changes and reduction in yield of more than 50% for most of the crops (Boyer 1982; Bray *et al.*, 2000; Wang *et al.*, 2003). Soil salinity alone can lead to a significant decrease in the yield, affecting as much as 7% of the world's arable land (Hasegawa *et al.*, 2000; Zhu, 2003). Furthermore, increased salinization is expected to reduce agricultural land by an estimated 30% by the year 2025 and up to 50% by the middle of the 21st century (Wang *et al.*, 2003). Therefore, it is extremely important to characterize the plant responses to abiotic stresses and identify gene(s) that may be useful in improving tolerance to such stresses.

A version of this chapter has been published and a provisional patent is filed: ***Srivastava S.****, Rahman H., Shah S. and Kav N.N.V. (2006) Constitutive expression of the pea ABA-responsive (ABR17) cDNA confers multiple stress tolerance in Arabidopsis thaliana* ***Plant Biotechnology Journal*** *4, 529-549.*

The availability of high throughput genomics and proteomics technologies enables us to conduct a comprehensive study of the genome, the transcriptome and the proteome under different abiotic and biotic stress conditions (Ramonell and Somerville, 2002; Agrawal *et al.*, 2005). Previously, an analysis of salinity-induced changes in the pea root proteome revealed an increase in the levels of several members of a PR protein family (PR 10; Kav *et al.*, 2004), including PR 10.1 and the ABA-responsive protein ABR17 (PR 10.4). The PR proteins are part of a multicomponent defense response in many plants, which respond to various abiotic and biotic stresses (Walter *et al.*, 1990). They are grouped into 14 different families based on their serological relations, homology at the nucleotide/amino acid sequence level, and similarities in biological functions (van Loon *et al.*, 1994; Van Loon and Van Strien, 1999).

Proteins belonging to the PR 10 family have been detected in a variety of anthophytes, monocots as well as dicot plants (Biesiadka *et al.*, 2002). PR 10 proteins are small (15–18 kDa), acidic and intracellular, unlike other PR proteins that are extracellular (Walter *et al.*, 1990; van Loon *et al.*, 1994). These intracellular PR (IPR) proteins were first described in cultured parsley cells upon elicitor treatment (Somssich *et al.*, 1988) and as described earlier, have subsequently been detected in many species. *PR 10* genes are known to be induced by pathogens (Fristensky *et al.* 1985; McGee *et al.* 2001; Borsics and Lados 2002), as well as other stresses such as salinity, drought, wounding and darkness (Osmark *et al.* 1998; Hashimoto *et al.*, 2004; Kav *et al.*, 2004). In addition to the induction by various stimuli, PR 10 proteins occur in high concentrations in roots, flowers and pollen (Biesiadka *et al.*, 2002 and references therein). These observations suggest that in addition to their role(s) in the plant stress response, they could play an important role during the normal growth and development of plants (Wu *et al*., 2003).

The pea ABA-responsive protein ABR17 is similar to pea disease resistance response proteins; it is produced late in seed development, and is induced by exogenous application of ABA (Iturriaga *et al.*, 1994; Colditz *et al.*, 2004). Many proteins with significant homology to the pea ABA-responsive protein, including an IPR protein from bean (Walter *et al.*, 1990), garden pea (Fristensky *et al.*, 1988), parsley (Somssich *et al.*, 1988), major birch pollen allergen Bet v1 (Breiteneder *et al.*, 1989), potato (Constabel and Brisson, 1992), and SAM22 from soybean (Crowell *et al.*, 1992). Several gene products homologous to ABR17, proteins also known as dehydrins and late embryogenesis abundant (LEA) related proteins, have been identified in different plant systems (Skriver and Mundy, 1990; Close *et al.*, 1993; Goday *et al.*, 1994).

Despite the identification of proteins with significant similarities to ABR17 (and other PR 10) in many species, a direct role for these proteins in mediating plant responses to abiotic stresses has not been convincingly demonstrated. We have reported previously (Srivastava *et al.*, 2004) that the constitutive expression of a *PR 10* (*PR 10.1*) gene from pea in *Brassica napus* ameliorates the effects of salinity stress during germination and early seedling growth. In this article, we demonstrated that *ABR17* (classified as a member of the *PR 10* family in pea) is able to enhance germination as well as tolerance of *A. thaliana* under multiple abiotic stresses. Furthermore, we compared the proteomes of *ABR17* transgenic and wild type seedlings in order to probe the mechanisms underlying the enhanced stress tolerance. Our findings are discussed within the context of the known biological activities of PR 10, as well as its potential utility in engineering multiple stress tolerance in plants.

5.2. Materials and methods

5.2.1. Plant expression vectors

The pea *ABR17* cDNA clone was kindly provided by Dr. Trevor Wang, Department of Metabolic Biology, John Innes Centre, Norwich, UK. The cDNA was amplified using the forward (5'-GTGGTCG**AAGCTT**ATGGGTGTCTTTGTTTTTGATGATGAATAC-3') and (5'-TATATAG**CTCGAG**TTAGTAACCAGGATTTGCCAAAACGTAACC-3') reverse primers, respectively. The restriction enzyme sites Hind III and Xho I are highlighted on the forward and reverse primers, respectively. The amplified cDNA fragment was ligated between the CaMV35S promoter and the rbcS3' terminator in the binary vector pKYLX71 (Schald *et al.*, 1987), and the resulting gene construct was sequenced to ensure ligation of the cDNA in the correct orientation and the absence of any mutations/rearrangement.

The gene construct was subsequently introduced into the disarmed *Agrobacterium tumefaciens* strain GV3101 through a triparental mating technique using *E. coli* strain HB101 carrying the helper plasmid pRK2013. Transconjugant *A. tumefaciens* was selected on solid medium containing rifampicin, gentamicin and tetracycline. Plasmid DNA was extracted from *A. tumefaciens* and analyzed by restriction digestion to ensure that no rearrangements had taken place during the introduction into this bacterium.

5.2.2. Transformation of *Arabidopsis thaliana*

A. thaliana ecotype WS was transformed using the floral dip method (Clough and Bent, 1998). Briefly, plants were grown in growth chambers at 25°C until flowering with a 16-h light/8-h dark photoperiod. Primary racemes were clipped to encourage proliferation of secondary bolts. *A. tumefaciens* cells at mid-log stage of growth were centrifuged and resuspended to a density of 0.8 (Abs_{600}) in 5% sucrose solution containing 0.05% silwet L-77. Above-ground parts of *A. thaliana* plants were dipped in this *Agrobacterium* solution with gentle agitation. Dipped plants were covered with plastic wrap (for 16-24 h) to maintain high humidity and were returned to growth chambers when the seeds were ready for harvest. Dry seeds (T_0) were surface sterilized and placed on 1/2 strength MS plates containing 50 mg/L kanamycin. The seeds were cold treated at 4°C for at least 2 d, and grown at 25°C with 16 h light/8 h dark, 100 μmol $m^{-2}s^{-1}$ light for 7-10 d. Plants that survived on the kanamycin plates were transferred to soil and maintained in growth cabinets. A number of independent transgenic lines were tested for their abilities to germinate on MS plates containing 75 or 150 mM NaCl, and those with enhanced germination were selected for the production of homozygous plants. Seeds from the transgenic plants

(T_1 seeds) were screened on kanamycin plates to determine segregation ratios and those with a monogenic (3:1) segregation ratio and exhibiting enhanced germination in the presence of NaCl were used for homozygous T_2 seed production. Bulked homozygous seeds from T_2 plants were used in all subsequent experiments.

5.2.3. RT-PCR analysis

Total RNA, isolated from wild type and transgenic seedlings, was reverse transcribed and subsequently amplified to detect *ABR17* transcripts. RNA was isolated using the QIAGEN RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) from pooled 2-week-old seedling tissue. The isolated RNA was treated with RNase-free DNase (Qiagen) to ensure the complete removal of DNA prior to RT-PCR. Reverse transcription and first strand cDNA synthesis of total RNA (50 ng) was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). PCR reactions were carried out using the newly synthesized cDNA (2 µL) as the template and primers (forward: 5'-GGTGATCAAGGAAGCACAAGG-3' and reverse: 5'-TTTGGCCTTTGTTTCATCACG-3') specific to the pea *ABR17* coding sequence using a PCR Master Mix (Promega, Madison, WI, USA). The *ABR17* transcript was amplified using the following thermocycling parameters: 94°C, 2 min; 35 cycles at 94°C, 1 min; 62°C, 1 min; 72°C, 1 min; and a final extension of 72°C, 10 min. Plant *18s rRNA* primers (forward: 5'-CCAGGTCCAGACATAGTAAG-3' and reverse: 5'-GTACAAAGGGCAGGGACGTA -3'; Duval *et al.*, 2002) were used as an internal control. PCR products were separated by electrophoresis on a 1.2% agarose gel and visualized under UV light after staining with ethidium bromide.

5.2.4. Western blot analysis

Two week-old pooled *Arabidopsis* seedlings were crushed in liquid N_2 and 500 µL extraction buffer (0.5 M Tris-HCl, pH 6.8; containing 10% glycerol; 10% SDS and 60 mM DTT) was added to 200 mg ground tissue. The tubes were vortexed and a small amount of protamine sulfate on the tip of a spatula was added and incubated at RT for 15 min prior to centrifugation at 12,500 g for 15 min. Ice-chilled acetone containing 0.07% DTT (5 equal volumes) was added to the supernatants and they were centrifuged once more as described above. The pellets were dried for 15 min under vacuum and resuspended in 100 µL of 50 mM Tris pH 6.8 containing 0.5% SDS. The concentration of protein in the resuspended samples was determined using a modified Bradford assay and the samples stored at -20ºC until subjected to Western blot analysis.

Protein (30 µg) was applied to polyacrylamide gels (15%) and subjected to electrophoresis according to Laemmli (1970) using a Mini PROTEAN 3 vertical slab system (Bio-Rad) at constant 160 V until the dye front reached the bottom of the gel. After electrophoresis the gel was equilibrated in transfer buffer (48 mM Tris, pH 9.2 containing 39 mM glycine, 20% methanol and 1.3 mM SDS) for 15 min and transferred to polyvinylidene fluoride (PVDF) membrane for 25 min at 15 V using Trans Blot SD, semi-dry transfer apparatus (Bio-Rad). Following transfer, the membrane was blocked with TBS (10 mM Tris-HCl, pH 7.5; 150 mM NaCl) containing 5% non-fat skim milk powder for 2 h and rinsed with TTBS (TBS containing 0.05% Tween 20) for 10 min. The primary antibody solution (rabbit anti-PR 10.4 in TTBS; 1:20,000) was added to the membrane and, after 1 h incubation, the membrane was washed 3 times for 5 min each with TTBS. The membrane was then incubated with secondary antibody solution [goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Abcam, Cambridge, MA, USA) in TTBS; 1:10,000] for 1 h after which the membrane was washed 3 times for 5 min each with TTBS followed by a 5 min wash with TBS. Bands on the blot were visualized by staining the membrane with TMB peroxidase substrate kit (Vector laboratories Inc., Burlingame, CA, USA) according to manufacturer's instructions.

5.2.5. Plant growth and germination experiments

Wild type and transgenic lines (6.9, 14.9, 25.20) of *A. thaliana,* ecotype WS seeds were grown on ½ strength MS medium containing 1.5% sucrose, 0.8% agar, pH 5.7 and various salt concentrations (0, 75 and 150 mM NaCl) in 25 x 100 mm Petri dishes. Seeds were surface sterilized by immersing in 70% ethanol for one min followed by rinsing twice with sterile deionized water. The seeds were then placed in a solution of 20% bleach for 15 min with occasional mixing, after which they were rinsed four times (five min each) with sterile deionized water. The experiment was designed in a randomized complete block design (RCBD) and at least five plates per treatment and 14 seeds per plates were used in these experiments. The plates were incubated at 22±1°C under continuous fluorescent light 30 μmol m^{-2} s^{-1} or at 10°C in a growth chamber (Conviron, model PGR15, Winnipeg, Manitoba) with coolwhite supplemented with incandescent bulbs 250 μmol m^{-2} s^{-1}. Germination of seeds was monitored every day and the numbers of germinated seeds were recorded daily for two weeks. Those seeds where the radicles had emerged were considered to have germinated. Each experiment was repeated at least three times.

5.2.6. Imposition of temperature stresses

Wild type and transgenic *Arabidopsis* lines (6.9, 14.9 and 25.20) were evaluated for tolerance to cold and high temperature stresses as described by Kim *et al.*, 2004. *Arabidopsis* seedlings were grown in MS plates for two weeks at 22±1°C under continuous fluorescent light 30 μmol m^{-2} s^{-1} and for the imposition of cold stress open MS plates containing seedlings were placed at -5°C for 4 h in dark after which the plates were returned to room temperature and photographed after 24 h of recovery. Similarily, *Arabidopsis* seedlings were grown in MS plates for two weeks at 22±1°C under continuous fluorescent light 30 μmol m^{-2} s^{-1} and for the imposition of heat stress open MS plates containing seedlings were placed in an dark incubator set at 48°C for 2 h after which they were returned to room temperature and photographed after 1 and 3 days of recovery.

5.2.7. Measurement of chlorophyll, carotenoid and ion-leakage

The chlorophyll and carotenoid concentrations, as well as ion-leakage, were used to assess plant damage due to the stresses. Total chlorophyll and carotenoid was extracted from pooled tissue of 2-week-old plants grown on MS plates, using a procedure modified from Kirk and Allen (1965). Pooled leaf tissue (0.05 g) was homogenized in 5 mL of ice-cold 80% acetone (v/v) and incubated at room temperature for 10 min in the dark. Samples were then centrifuged at 2,500 g for 15 min. The supernatant was decanted and absorbance measured at 663, 645 and 480 nm. Total chlorophyll (a+b) was estimated using a nomogram (Kirk, 1968) and the carotenoid levels were calculated using the formula (Kirk and Allen, 1965): $\Delta ACAR_{480} = \Delta A_{480} + 0.114\ \Delta A_{663} - 0.638\ \Delta A_{645}$, where A is absorbance and CAR is carotenoid content.

Membrane damage was assessed by measuring ion-leakage from cold-stressed leaves (Vettakkorumakankav *et al.*, 1999). Leaves from control and stressed plants were incubated in 20 mL of distilled water and agitated at room temperature for an hour following which the conductivity of the solution (initial value) was determined using a conductivity meter (HI 8733, Hanna Instrument, Woonsocket, RI, USA). The solutions were incubated at 4°C overnight and subsequently autoclaved to release total ions and the final conductivity values were determined. Ion leakage is expressed as a percentage of the initial to final values.

5.2.8. Two-dimensional electrophoresis

Protein extracts for two-dimensional electrophoresis were prepared according to the method described by Subramanian *et al.* (2005) with some modifications. Wild type and transgenic (6.9) seedlings (2-week-old) from MS plates were homogenized to a fine powder in liquid nitrogen. Homogenized tissue (0.3 g) was further homogenized in acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% DTT, transferred to eppendorf tubes and the volume was adjusted to 1.5 mL with acetone containing 10% (w/v) TCA and 0.07% DTT. Samples were incubated at -17°C for 1 h, centrifuged at 13,000 g for 15 min and the supernatants discarded. The pellets were washed by resuspending them in ice-cold acetone containing 0.07% DTT and centrifuged as described above. This step was repeated four additional times, the pellets were dried at room temperature in a speedvac for 30 min and resuspended in 400 μL Rehydration/Sample buffer (Bio-Rad, Mississauga, ON, Canada) containing 8 M urea, 2% w/v CHAPS, 40 mM DTT, 0.2% Bio-lyte 3-10 and 3 μL of 200 mM tributylphosphine (TBP). The samples were mixed vigorously, incubated overnight at 4°C and centrifuged at 4°C for 15 min at 13,000 g. The supernatants were placed in fresh tubes and protein concentrations determined using a modified Bradford assay (Bio-Rad) with BSA as the standard.

Two-dimensional electrophoresis of protein extracts was performed as previously described (Subramanian *et al.*, 2005). Briefly, immobilized pH gradient (IPG) strips (17 cm, Bio-Rad) were passively rehydrated overnight with 300 μg of protein in 300 μL of Rehydration buffer (8 M urea, 2% CHAPS, 40 mM DTT, 0.2%

Bio-Lyte and 2 mM TBP). Isoelectric focusing (IEF) was performed using a Bio-Rad PROTEAN IEF unit to provide an optimum, maximum field strength of 600 V/cm and a 50 μA limit/IPG strip at 10,000 V for 60,000 Volt Hours (VH). Prior to second dimension separation, proteins in the rehydrated strips were reduced by incubating them twice in a solution (5mL/ strip) containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl, pH 8.8, 20% glycerol, and 130 mM DTT for 10 min each. The strips were then incubated in the above solution, but containing 135 mM iodoacetamide instead of DTT, twice for 10 min each in order to alkylate the reduced proteins. SDS-PAGE separation of proteins was performed on 13% polyacrylamide gels (20 X 20 cm, 1 mm thickness) using a PROTEAN II XI system (Bio-Rad) at a constant voltage (90 V) until the dye front reached the bottom of the gel. Protein spots were visualised using a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA) according to the instructions provided. Two-dimensional electrophoresis was performed at least three times with the extracted protein samples.

5.2.9. Image analysis

Images of the two-dimensional gels were acquired using a GS-800 calibrated densitometer (Bio-rad) and analyzed using PDQuest software (Bio-rad). Three gels each of wild type and transgenic (6.9) samples were used to generate the match-sets and individual spots were matched using the automated detection and matching feature of the software followed by manual refinements in order to eliminate artifacts and include spots that were missed by the automated detection process. In order to identify the protein spots whose levels were significantly different between the transgenic and wild type seedlings, the match sets from three replicate gels of wild type and line 6.9 protein samples were analyzed using Student's *t*-test feature of PDQuest software as described by the manufacturer. Those spots which were reproducibly altered in all three replicates and exhibited significant ($P<0.01$) differences were excised from the gels using sterile scalpels, and were subjected to electrospary ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS/MS) analysis.

5.2.10. ESI-Q-TOF MS/MS analysis

Tandem MS was performed at the Institute for Biomolecular Design, University of Alberta, on protein extracted from isolated gel spots as previously described (Subramanian *et al.*, 2005). Briefly, gel pieces were de-stained, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with 6 ng/μL trypsin (Promega Sequencing Grade Modified) in 50 mM ammonium bicarbonate (25 μl), for 5 h at 37°C in a fully automated fashion on a Mass Prep Station (Micromass, Manchester, UK). The tryptic peptides were subjected to LC/MS/MS analysis on a Micromass Q-ToF-2 mass spectrometer (Micromass) coupled with a Waters CapLC capillary HPLC (Waters Corp., Milford, MA, USA). Peptides were separated on a PicoFrit capillary reversed-phase column (5 μ BioBasic C18, 300 Angstrom pore size, 75 μ ID x 10 cm, 15 μ tip) (New Objectives, Woburn, MA, USA), using a linear water/acetonitrile gradient (0.2% formic acid), after desalting on a 300 μ x 5 mm

PepMap C18 column (LC Packings, Sunnyvale, CA, USA). Eluent was introduced directly to the mass spectrometer by electrospray ionization at the tip of the capillary column and data dependent MS/MS acquisition was performed for peptides with a charge state of 2 or 3. Proteins were identified from the MS/MS data by searching the NCBI non-redundant database with Mascot Daemon (Matrix Science, London, UK) including carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide as search parameters.

5.2.11. Statistical analysis

The experiment was designed in a randomized complete block design (RCBD). Two way analysis of variance (ANOVA) was performed using the mixed model procedure of SAS version 8e (Statistical Analysis System, 1985, SAS Institute, Cary, NC, USA). Mixed model was performed on individual experiments considering genotypes as fixed effect and block as random effect. An estimate of least square means and pooled standard errors were calculated.

5.3. Results

5.3.1. Generation of transgenic *Arabidopsis* plants and confirmation of gene expression

Transgenic *A. thaliana* plants were generated using the floral dip method (Clough and Bent, 1998). Seeds from T_0 plants (T_1 seeds) of 33 independent transgenic lines were selected and screened for their ability to germinate in the presence of 75 and 150 mM NaCl. The number of copies of the ABR17 cDNA in these lines was estimated based on their segregation on kanamycin plates. Three transgenic lines (6.9, 14.9 and 25.20) with single insertions of the *ABR17* cDNA that showed best germination in preliminary experiments under saline conditions were selected for the production of homozygous lines and further characterization.

The expression of the transgene was verified using *ABR17*-specific primers and along with *18s rRNA* primers as an internal control. The presence of an *ABR17*-specific transcript was confirmed by this RT-PCR analysis, which produced the expected 319-bp amplification product, which was absent in the wild type as well as the negative control (Figure 5-1A). The quality of mRNA, cDNA and the amplification reactions was confirmed by the successful amplification of the expected *18s rRNA* product in all samples (Figure 5-1A). The presence of the ABR17 protein in all the three transgenic lines was confirmed by Western blot analysis with PR 10.4-specific polyclonal antibodies. Protein extracts prepared from the transgenic plants revealed the presence of a strongly reacting band with the molecular weight corresponding to that of ABR17, whereas extracts from the wild type plants did not show the presence of this band (Figure 5-1B).

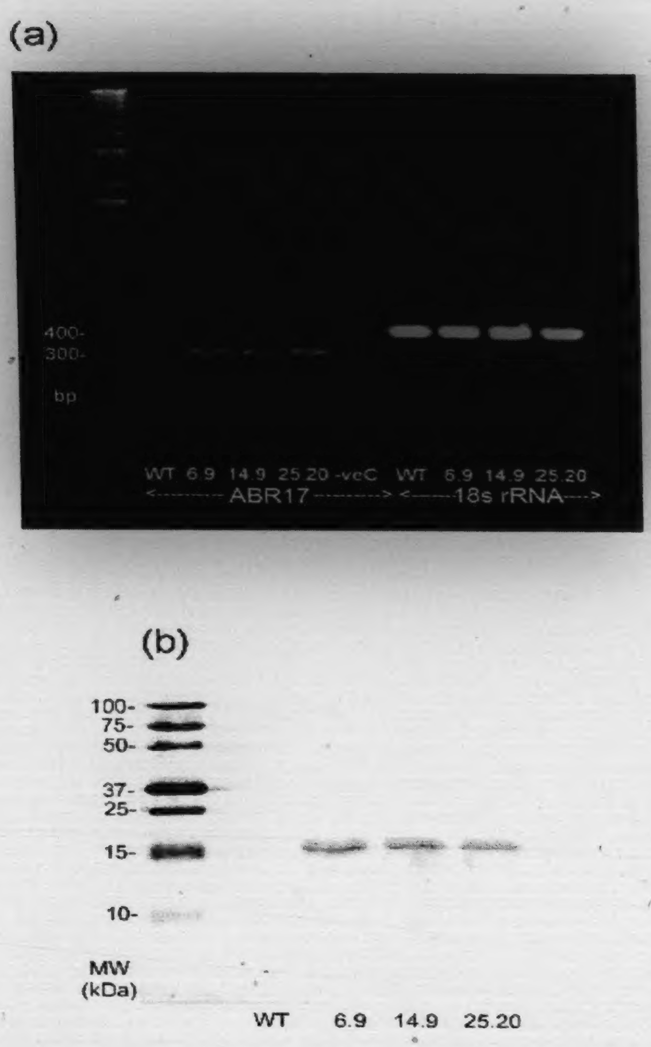

(a)
400-
300-
bp
WT 6.9 14.9 25.20 -veC WT 6.9 14.9 25.20
<---------- ABR17 ----------> <------18s rRNA---->
(b)
100-
75-
50-
37-
25-
15-
10-
MW
(kDa)
WT 6.9 14.9 25.20

Figure 5-1. Expression of *ABR17* cDNA in transgenic *A. thaliana*. (a) RT-PCR analysis of *ABR17* and *18s RNA* expression demonstrating the presence of *ABR17* transcript in the 3 transgenic lines (6.9, 14.9, 25.20) and its absence in the wild type (WT) as well as the presence of 18s transcript throughout. (b) Western blot analysis of protein extracts (from 2 week-old seedlings) demonstrating the presence of a unique band corresponding to the molecular weight of ABR17 in the three transgenic lines.

5.3.2. Effects of salinity and cold on germination

The ability of the three transgenic lines to germinate in the presence of NaCl was evaluated at room temperature and 10ºC and the results are shown in Figure 5-2. At room temperature, in the absence of NaCl, we observed 100% germination in the wild type as well as the three transgenic lines. At the lower temperature (10ºC), while the germination in all the lines was not 100%, there were no significant differences in the numbers of germinated seeds on any day (Figure 5-2C). However, the transgenic seedlings germinated earlier in the presence of 75 and 150 mM NaCl at both temperatures tested (Figure 5-2). For instance, the transgenic lines 6.9 and 14.9 initiated germination by day 1 in the presence of 75 mM NaCl, whereas no wild type or 25.20 transgenic seeds had germinated at this point (Figure 5-2A). Similarly, in the presence of 150 mM NaCl, all three transgenic lines had initiated germination by day 3 at room temperature, whereas no significant germination in the wild type had occurred at this time (Figure 5-2B).

Figure 5-2. Effects of NaCl and low temperature on the germination of wild type and *ABR17* transgenic seedlings. Effects of (a) 75 and (b) 150 mM NaCl, respectively at room temperature. Effects of (c) 0, (d) 75 mM and (e) 150 mM NaCl, respectively at 10ºC. Germination was recorded from at least 42 seeds in each biological replicate and experiment was repeated at least three times. An estimate of least square means and pooled standard errors are presented.

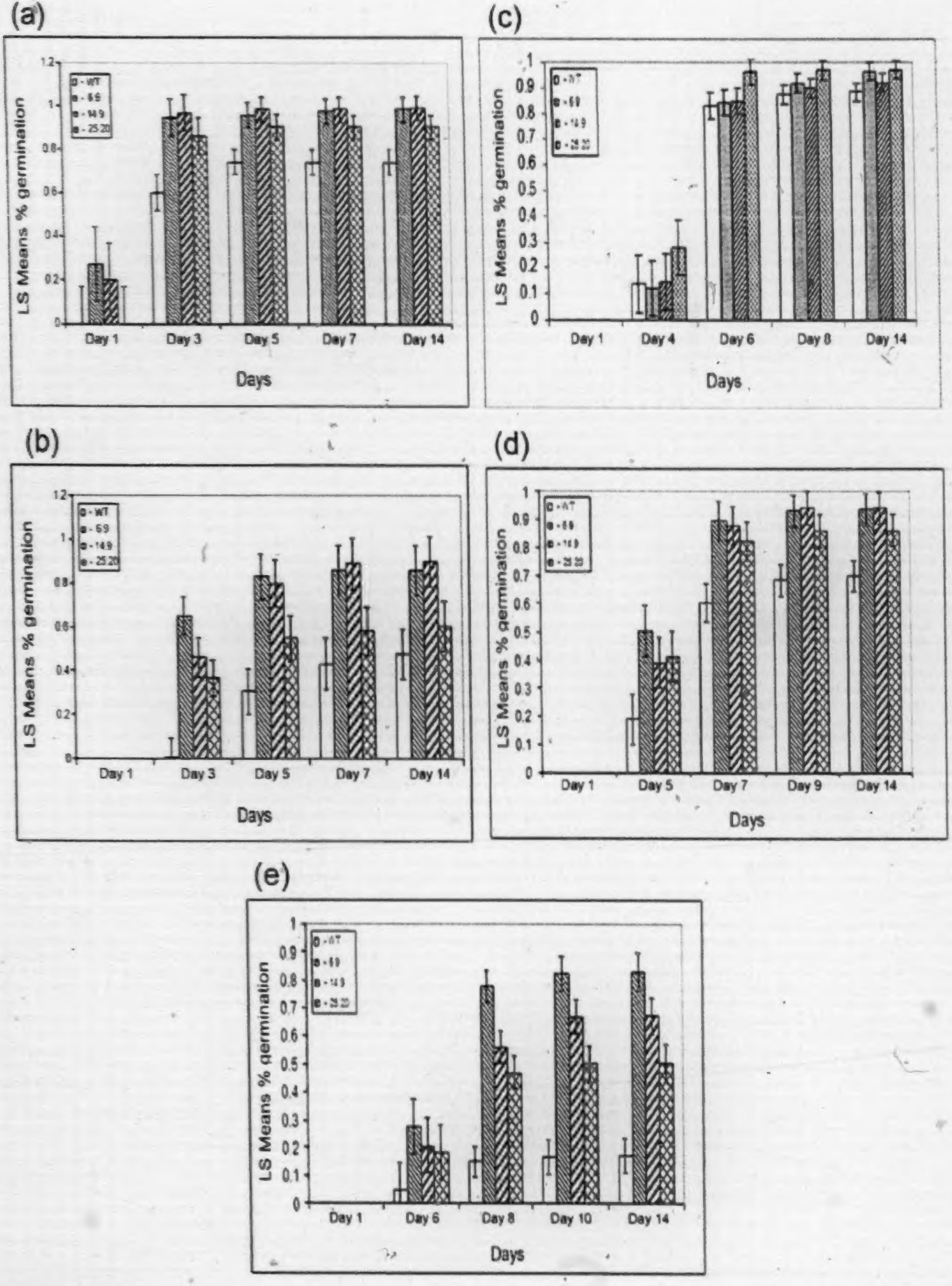

(a)
LS Means % germination
0
0.2
0.4
0.6
0.8
1
1.2
Day 1
Day 3
Day 5
Day 7
Day 14
Days
WT
6.9
14.9
25.20
(c)
LS Means % germination
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Day 1
Day 4
Day 6
Day 8
Day 14
Days
(b)
LS Means % germination
Day 1
Day 3
Day 5
Day 7
Day 14
Days
(d)
LS Means % germination
Day 1
Day 5
Day 7
Day 9
Day 14
Days
(e)
LS Means % germination
Day 1
Day 6
Day 8
Day 10
Day 14
Days

Figure 5-2.

The maximum germination observed at room temperature in the presence of 75 mM NaCl at the conclusion of the experiment (on day 14) in the wild type was ~74%, compared to that of 99%, 99%, 90% for the transgenic lines 6.9, 14.9 and 25.20, respectively (Figure 5-2A). In the case of 150 mM NaCl, the maximum germination in the wild type was 47% compared to 85%, 89% and 60% in the transgenic lines 6.9, 14.9 and 25.20, respectively (Figure 5-2B).

When the germination of the seeds was assessed at 10°C, in the presence of either 75 or 150 mM NaCl, the differences in germination rates of the transgenic seeds were obvious with the transgenic lines clearly performing better than the wild type (Figures 5-2D and E). At 10°C, on day 5, in the presence of 75 mM NaCl all three transgenic lines had significantly higher germination rates compared to the wild type, with the transgenic line 6.9 appearing to be the best among the transgenics (Figure 5-2D). The trend remained the same throughout the entire period of the experiment (14 days) for 75 mM NaCl treatments. At the end of the 14-day experiment, in the presence of 75 mM NaCl at 10°C, 70% of the wild type seeds had germinated, whereas the germination rates were 94%, 94% and 86% in the three transgenic lines 6.9, 14.9 and 25.20, respectively (Figure 5-2D). In the presence of 150 mM NaCl, the differences between the wild type and the three transgenics were apparent on day 6 and was very clear on day 8 (Figure 5-2E). At this salt concentration, only 17% of the wild type seeds had germinated by day 14, whereas the germination rate was 83%, 67% and 50% in the three transgenic lines 6.9, 14.9 and 25.20, respectively. Once again, the transgenic line 6.9 appeared to be better than the other two lines (Figure 5-

2E). All the observed differences between the germination rates of the transgenic and wild type seeds were statistically significant ($P < 0.05$).

5.3.3. Appearance of seedlings germinated and grown in the presence of NaCl

The appearance of the wild type and transgenic seedlings grown on MS plates containing 0, 75 or 150 mM NaCl at room temperature after one and two weeks is shown in Figures 5-3A and B, respectively. It is clear that there were no obvious differences between the wild type and 3 transgenic lines in the absence of salt after one week of growth (Figure 5-3A). However, after 2 weeks, in the absence of salt, it appears as though both roots and shoots of the transgenic seedlings were better developed compared to the wild type (Figure 5-3B). As previously described for the germination experiments, the differences between the wild type and transgenic seedlings were more apparent in the presence of NaCl (Figure 5-3), with the deleterious effects on root and shoot development being more pronounced at the higher (150 mM) NaCl concentration. It is clear from the appearance of the seedlings (Figure 5-3) that the deleterious effects of 150 mM NaCl at both 1 and 2 weeks in the transgenic lines were considerably less compared to those on the wild type seedlings. These observations were confirmed by evaluating the effect of the salinity treatments on the chlorophyll and carotenoids levels, which were measured in 2-week-old control as well as 75 mM NaCl-treated tissues.

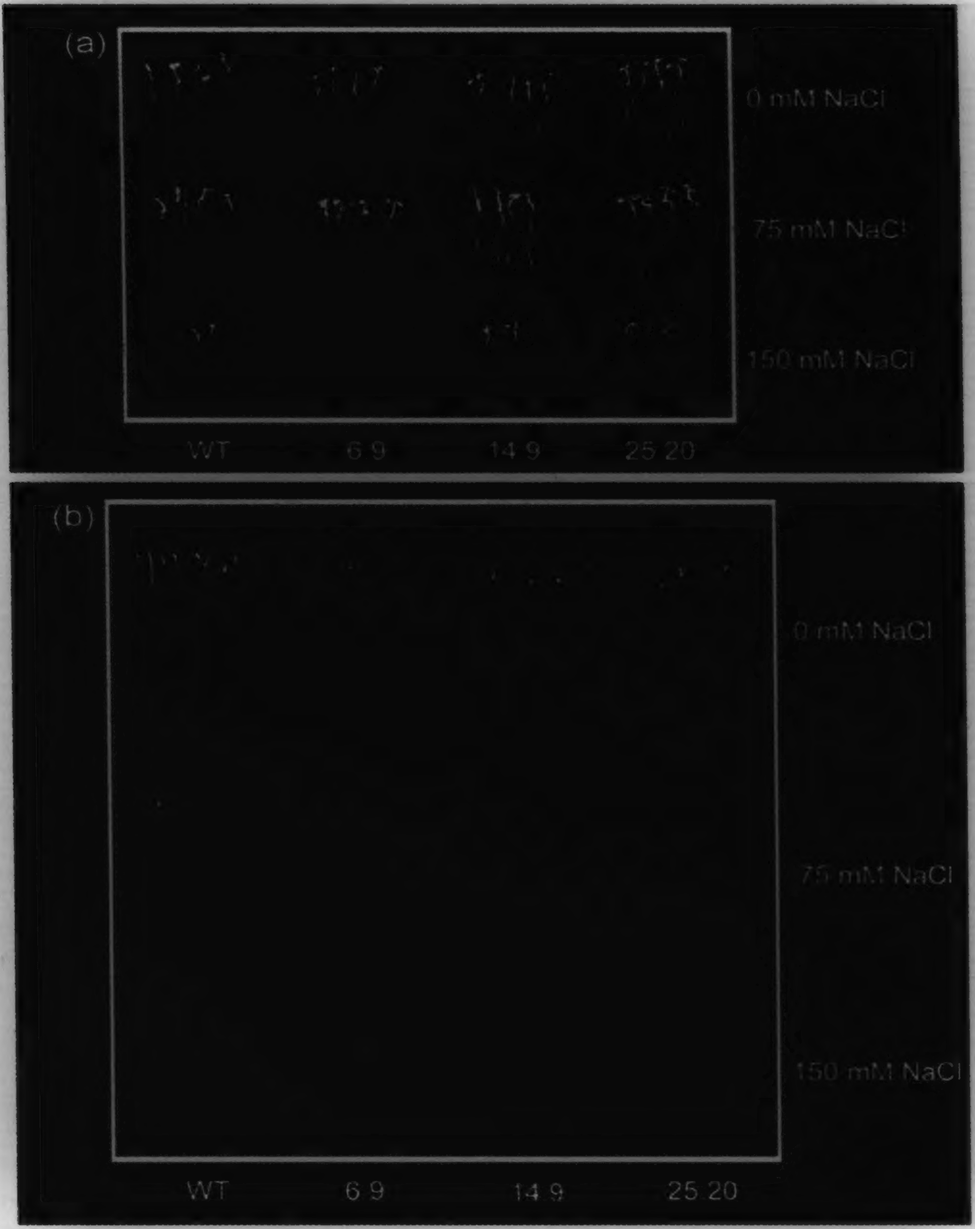
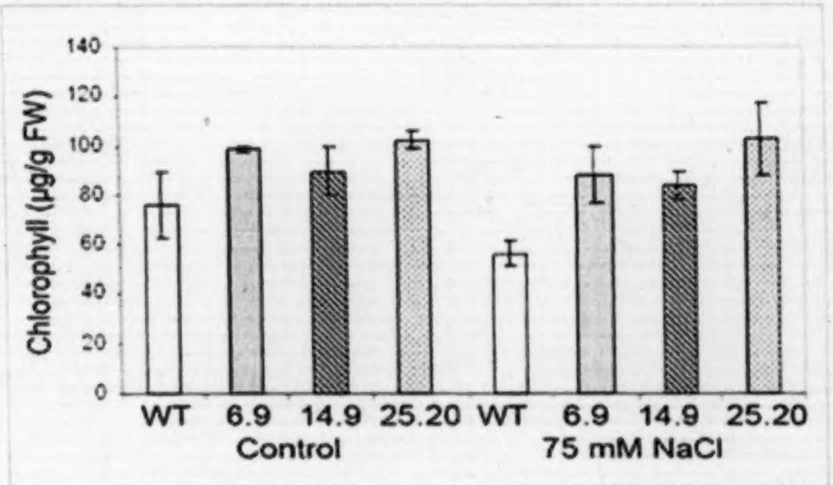

(a)
0 mM NaCl
75 mM NaCl
150 mM NaCl
WT
6 9
14 9
25 20
(b)
0 mM NaCl
75 mM NaCl
150 mM NaCl
WT
6 9
14 9
25 20

Figure 5-3. Effects of salinity on the seedlings germinated and grown at room temperature. Morphology of seedlings germinated after (a) 1 and (b) 2 weeks of growth at 0, 75 and 150 mM NaCl.

(c)



(d)

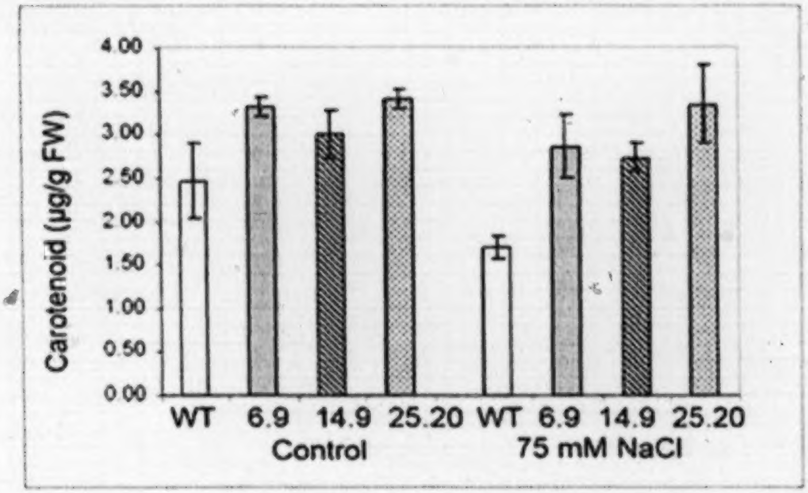


Figure 5-3 (contd). (c) Total chlorophyll and (d) carotenoid levels following two weeks of growth at 0 and 75 mM NaCl, mean ± standard errors are presented.

The chlorophyll levels were significantly (*P*<0.05) reduced in the wild type seedlings, whereas the levels in the transgenics grown in 75 mM NaCl were not significantly different from the untreated samples (Figure 5-3C). The effect of 75 mM NaCl was more pronounced on the carotenoid levels of the wild type seedlings, with the salt treatment reducing the carotenoid levels significantly (*P*<0.05) compared to the transgenics (Figure 5-3D). It is also apparent that the carotenoid levels in the untreated transgenic seedlings were higher compared to that of the untreated wild type seedlings (Figure 5-3D). These elevated levels of carotenoids may be contributing to the better tolerance to NaCl exhibited by the transgenic lines.

The appearance of seedlings after two and three weeks at 10ºC in the presence or absence of NaCl is shown in Figure 5-4. It is evident that at this lower temperature, in the presence or absence of NaCl, the transgenic seedlings appear to be healthier than the wild type after 2 (Figure 5-4A) and 3 weeks (Figure 5-4B) of growth, suggesting that they are able to better tolerate the lower temperature stress. Chlorophyll and carotenoid levels could not be measured in the seedlings subjected to the NaCl treatment at this lower temperature due to the lack of sufficient tissue to perform those experiments. Therefore, the appearance of the seedlings also support the data obtained from the experiments that assessed the abilities of the transgenic lines to germinate under these stress conditions.

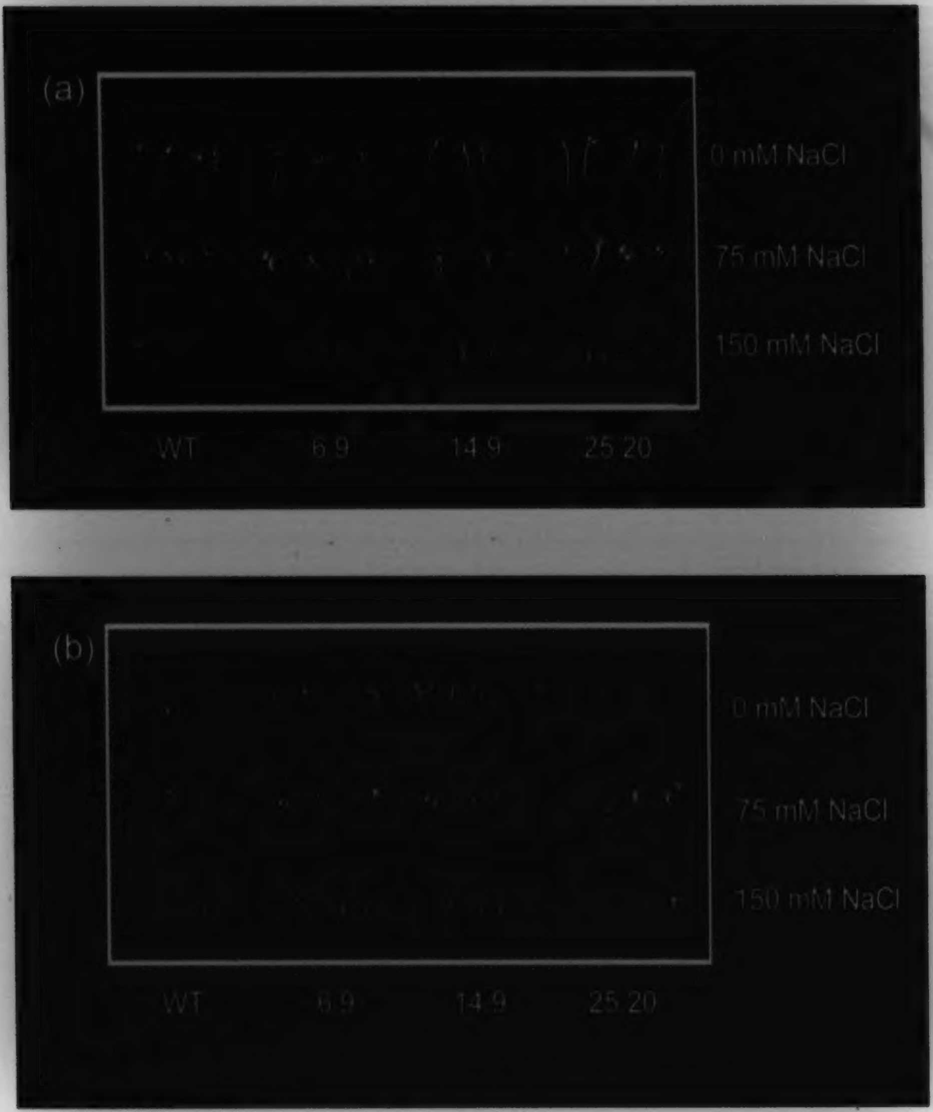

(a)
0 mM NaCl
75 mM NaCl
150 mM NaCl
WT
6.9
14.9
25.20
(b)
0 mM NaCl
75 mM NaCl
150 mM NaCl
WT
6.9
14.9
25.20

Figure 5-4. Effects of salinity on the seedlings germinated and grown at 10ºC. Morphology of seedlings after (a) 2 and (b) 3 weeks of growth at 0, 75 and 150 mM NaCl.

5.3.4. Assessment of tolerance to freezing temperature

In addition to the ability to germinate and grow under stress conditions, it is also important to enhance the ability of plants to tolerate sub-zero temperatures. We were therefore interested in evaluating whether these transgenic lines were capable of tolerating exposure to freezing temperature (-5°C). The appearance of plants grown on MS plates 24 h after a 4 h treatment at -5°C is shown in Figure 5-5. From the appearance of the plants 24 h after returning to room temperature, it is apparent that almost all the wild type plants had collapsed, whereas the transgenic lines appeared healthier indicating that these lines were more tolerant to the imposed stress.

In order to confirm that this was indeed the case, we determined the percent ion leakage in the wild type and transgenic seedlings after 4 h of stress and 4 h of incubation at 4°C as well as after the 24 h recovery at room temperature (Table 5-1). The percent ion leakage, which is indicative of membrane damage, after the stress and 4°C incubation, was significantly less in all three transgenic lines compared to the wild type, even though it appears as if line 6.9 suffered the least amount of membrane damage when compared to the other two transgenic lines (Table 5-1). These observations are also supported by the ion leakage observed after the 24 h recovery period where, once again, it was observed that line 6.9 had the least amount of membrane damage (Table 5-1).

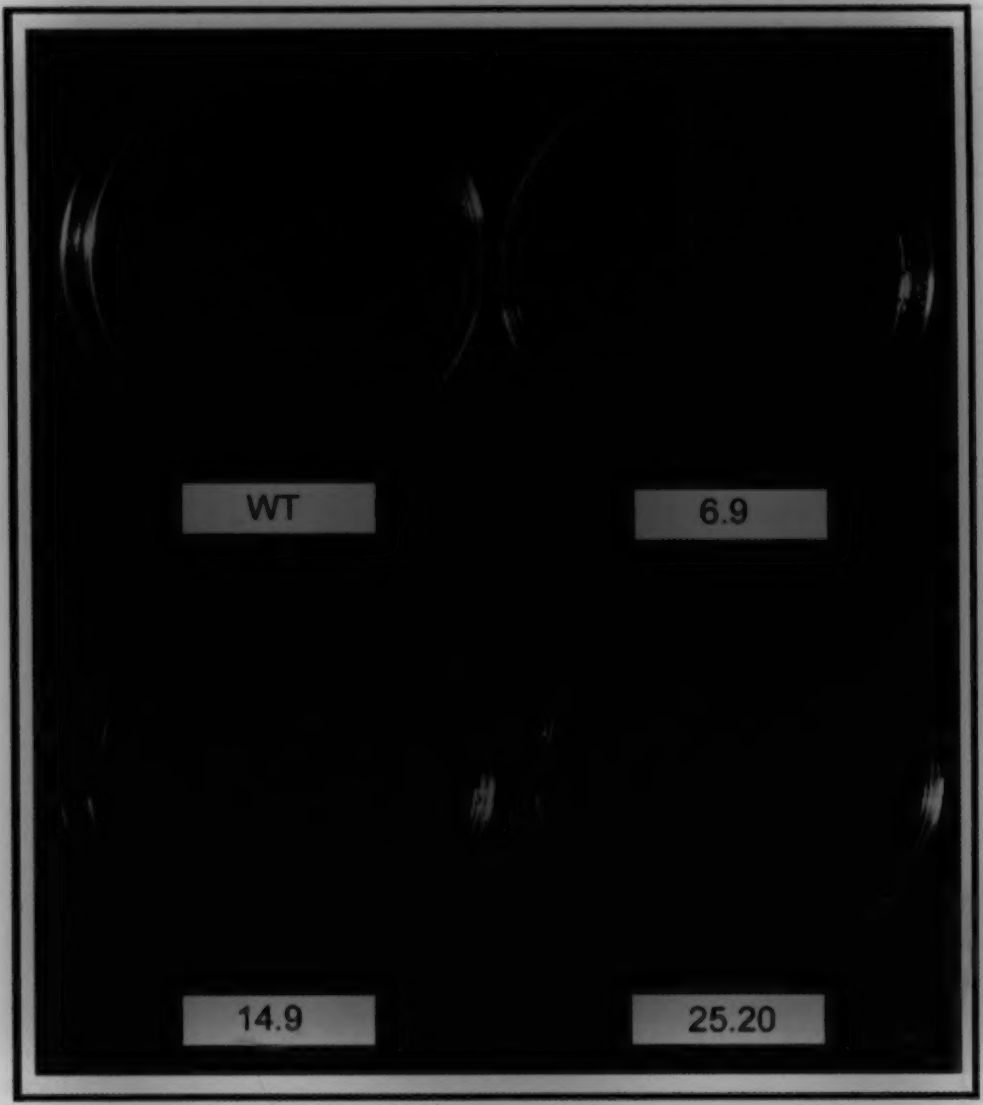


Figure 5-5. Freezing-tolerance of *ABR17* transgenic plants. Appearance of seedlings following 1 day of recovery after exposure to cold stress (-5°C) for 4 h. Observations were recorded from at least 28 seeds in each biological replicate and experiment was repeated at least three times.

Table 5-1. Effects of cold stress on ion leakage, chlorophyll and carotenoid concentration.

Percentage Ion leakage Cold stress -5°C for 4 h and 4 h incubation at 4°C Mean ± SE	
WT	61.69 ± 3.32
6.9	37.17 ± 3.66
14.9	47.22 ± 1.49
25.20	44.84 ± 1.17
Percentage Ion leakage 1 day recovery after cold stress -5°C for 4 h	
WT	51.36 ± 4.17
6.9	38.26 ± 4.41
14.9	44.57 ± 6.94
25.20	38.31 ± 7.40
Chlorophyll (µg/g FW) 1 day recovery after cold stress -5°C for 4 h	
WT	72.45 ± 1.02
6.9	98.47 ± 18.93
14.9	81.83 ± 4.29
25.20	74.41 ± 15.64
Carotenoid (µg/g FW) 1 day recovery after cold stress -5°C for 4 h	
WT	2.50 ± 0.05
6.9	2.95 ± 0.56
14.9	2.68 ± 0.16
25.20	2.37 ± 0.56

Even though the appearance, as well as the ion leakage data, indicated that the transgenic plants were significantly more tolerant to the cold stress, there were no significant differences in the chlorophyll or carotenoid content when measured after the stress as well as after the recovery period (Table 5-1).

The enhanced germination of the transgenic lines in the presence of NaCl and the increased tolerance of these lines to cold temperature stress prompted us to test whether these *ABR17* transgenic plants would exhibit enhanced tolerance to heat stress. Wild type and transgenic plants (2-week-old) were exposed to 48°C for 2 h and allowed to recover for 1 day at room temperature. The appearance of these plants after the 1 day recovery period is shown in Figure 5-6. It is evident that all 3 transgenic lines appeared healthier compared to the wild type after 1 day of recovery suggesting that *ABR17* expression could confer multiple stress tolerance. However, we did not investigate the heat tolerance of these transgenic lines in greater detail as it was beyond the scope of this study.

WT
6.9
14.9
25.20

Figure 5-6. Heat-tolerance of *ABR17* transgenic plants. Appearance of seedlings following 1 day of recovery after exposure to heat stress (48°C) for 2 h. Observations were recorded from at least 28 seeds in each biological replicate and experiment was repeated at least three times.

5.3.5. Characterization of proteome-level differences between wild type and transgenic *A. thaliana*

In order to probe the physiological basis for the observed enhancement of stress tolerance in transgenic *A. thaliana* seedlings expressing the pea *ABR17* gene, we performed two-dimensional electrophoresis to compare proteome-level changes brought about by the transgene expression. We selected the transgenic line 6.9 for these comparisons because of the fact that it exhibited the highest degree of stress tolerance. Representative images of two-dimensional gels obtained from wild type and line 6.9 protein extracts are shown in Figure 5-7. The intensities of 24 protein spots were observed to be significantly ($P<0.01$) and reproducibly altered in the transgenic line compared to the wild type (Figure 5-7). Out of these 24 protein spots, the intensities of 7 were greater, 15 were lower and two spots were unique in the transgenic line (Figure 5-7). The identities of these 24 spots were established using tandem MS and are presented in Table 5-2. In all cases, the Mascot searches of the NCBI non-redundant database generated significant hits with scores above the threshold value and in most cases these were a result of multiple peptide matches (Table 5-2). These scores are based on individual ion scores where the ion score is -10*log (P) and P is the probability that the observed match is a random event. A score above the threshold value indicates sequence identity or extensive homology ($P<0.05$).

Among the proteins that were identified in this study, two were unique and the identities of both were established as pea ABR17 (spots 23 and 24; Figure 5-7; Table 5-2).

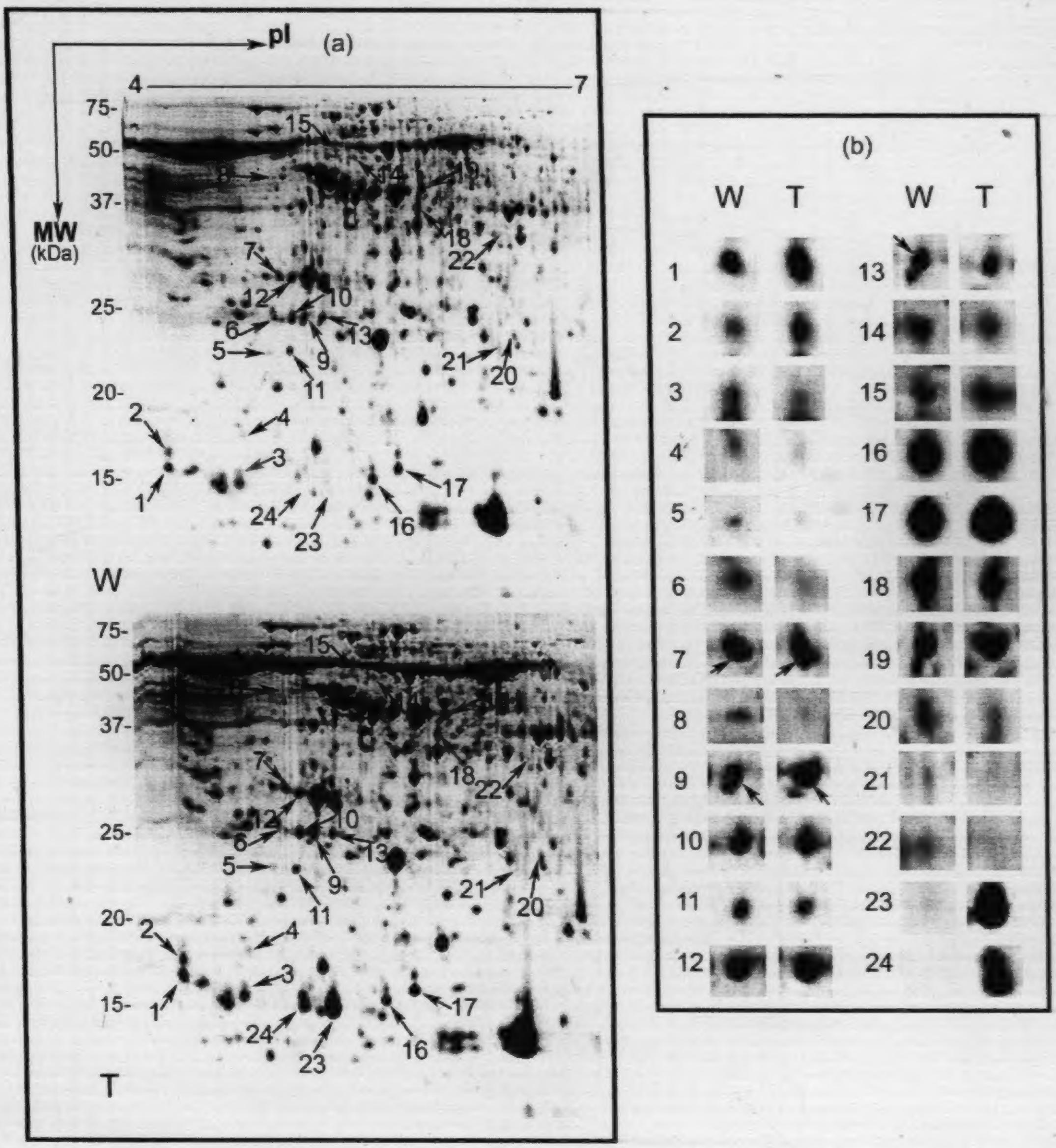

pI
(a)
4
7
MW
(kDa)
75-
50-
37-
25-
20-
15-
W
T
(b)
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Figure 5-7. Images of two-dimensional gels of protein extracted from wild type (W) and transgenic (T) plants. (a) Representative images of whole gels and (b) a closer view of changes in the intensities of the spots selected for identification.

Although only one protein corresponding to ABR17 was expected the fact that there are two suggests the possibility that the additional spot may be the result of post-translational modification. Most of the proteins identified in this study were associated with photosynthesis and primary metabolic pathways, including putative photosystem I subunit PSI-E protein (spot 2; Figure 5-7; Table 5-2), chlorophyll binding proteins (spots 6, 10 and 13; Figure 5-7; Table 5-2), Cp29 (spot 7; Figure 5-7; Table 5-2), PSBO-2/PSBO2; oxygen evolving protein (spot 12; Figure 5-7; Table 5-2), Ribose-5-phosphate isomerase (spot 9; Figure 5-7; Table 5-2), phosphopyruvate hydratase (spot 14; Figure 5-7; Table 5-2) and RUBISCO activase (spot 19; Figure 5-7; Table 5-2). Other interesting proteins identified in this study were DRT112 (spot 1; Figure 5-7; Table 5-2) which is thought to be involved in DNA damage repair, and glycine-rich proteins (spots 16 and 17; Figure 5-7; Table 5-2), which are involved in post-transcriptional gene regulation.

Table 5-2. Details of proteins identified by ESI-Q-TOF MS/MS

Spot No.	Protein identity	MS/MS ESI-Q-ToF			[c] Access. No.	Mr/pI	Fold change
		[a] PM %	[b] score	Sequence			
1	DRT112 [*Arabidopsis thaliana*]	14%	88 (>48)	NNAGYPHNVVFDEDEIPSGVDVAK	gi|166696	17089/ 5.06	3.49 ± 0.57 ↑
2	Putative photosystem I subunit PSI-E protein [*Arabidopsis thaliana*]	23%	153 (>49)	AAEDPAPASSSSK RESYWFK NVGSVVAVDQDPK	gi|24030202	14756/ 9.92	18.17 ± 10.23 ↑
3	GDCH [*Arabidopsis thaliana*]	15%	147 (>49)	FCEEEDAAH VKPSSPAELESLMGPK	gi|15226973	18050/ 5.24	0.14 ± 0.13 ↓
4	Unknown protein [*Arabidopsis thaliana*]	7%	85 (>42)	VEVTEAEVELGFK	gi|18422918	17603/ 4.80	0.68 ± 0.07 ↓
5	Bas1 protein [*Hordeum vulgare*]	27%	140 (>48)	YVILFFYPLDFTFVCPTEITAFSDR SGGLGDLKYPLVSDVTK EGVIQHSTINNLGIGR	gi|861010	23398/ 5.48	0.47 ± 0.02 ↓
6	LHB1B2; chlorophyll binding [*Arabidopsis thaliana*]	59%	483 (>49)	GPSGSPWYGSDR YLGPFSGEPPSYLTGEFPGDYGWDTA GLSADPETFAR WAMLGALGCVFPELLAR FGEAVWFK LAMFSMFGFFVQAIVTGK GPLENLADHLADPVNNNAWAFATNFVPGK YLGPFSGEPPSYLTGEFPGDYGWDTA GLSADPETFAR	gi|18403546	28093/ 5.28	0.42 ± 0.11 ↓
	LHB1B1; chlorophyll binding [*Arabidopsis thaliana*]	60%	445 (>49)	ASKPTGPSGSPWYGSDR YLGPFSGEPPSYLTGEFPGDYGWDTA GLSADPETFAR WAMLGALGCVFPELLAR	gi|18403549	28209/ 5.15	

				FGEAVWFK VAGDGPLGEAEDLLYPGGSFDPLGLA TDPEAFAELK LAMFSMFGFFVQAIVTGK GPLENLADHLADPVNNNAWAFATNFVPGK			
7	Cp29 [*Arabidopsis thaliana*]	27%	499 (>49)	VNAGPPPPK SSYGSGSGSGSGSGSGNR LYVGNLSWGVDDMALENLFNEQGK GFGFVTLSSSQEVQK AINSLNGADLDGR VSEAEARPPR	gi|681904	34602/ 5.23	0.86 ± 0.05 ↓
	Inorganic diphosphatase/ magnesium ion binding / pyrophosphatase [*Arabidopsis thaliana*]	22%	326 (>49)	VQEEGPAESLDYR VFFLDGSGK VSPWHDIPLTLGDGVFNFIVEIPK IVAISLDDPK HFPGTLTAIR IPDGKPANR	gi|15242465	33644/ 5.71	
8	Putative aspartyl protease [*Arabidopsis thaliana*]	9%	177 (>49)	GDLASESILLGDTK SSVSLVSQTLK VIYDTTQER LGIVGENCR	gi|12324588	47745/ 6.06	0.47 ± 0.10 ↓
	ribosomal protein S1 [*Spinacia oleracea*]	8%	151 (>49)	VSDIATVLQPGDTLK AEEMAQTFR IAQAEAMAR	gi|18060	45044/ 5.41	
9	Ribose-5-phosphate isomerase [*Arabidopsis thaliana*]	53%	727 (>49)	AVEAIKPGMVLGLGTGSTAAFAVDQIGK LLSSGELYDIVGIPTSK SLGIPLVGLDTHPR IDLAIDGADEVDPNLDLVK	gi|15229349	29401/ 5.72	1.46 ± 0.09 ↑

				EKMVEAVADK MVEAVADK FIVVADDTK VDGDGKPYVTDNSNYIIDLYFK FQGVVEHGLFLGMATSVIIAGK NGVEVMTK			
10	Chlorophyll a/b binding protein (LHCP AB 180) [*Arabidopsis thaliana*]	67%	516 (>49)	GPSGSPWYGSDR YLGPFSGESPSYLTGEFPGDYGWDTA GLSADPETFAR WAMLGALGCVFPELLAR FGEAVWFK VAGNGPLGEAEDLLYPGGSFDPLGLA TDPEAFAELK LAMFSMFGFFVQAIVTGK GPIENLADHLADPVNNNAWAFATNFVPGK	gi|16374	25036/ 5.12	0.46 ± 0.02 ↓
	LHB1B2; chlorophyll binding [*Arabidopsis thaliana*]	31%	376 (>49)	GPSGSPWYGSDR WAMLGALGCVFPELLAR FGEAVWFK LAMFSMFGFFVQAIVTGK GPLENLADHLADPVNNNAWAFATNFVPGK	gi|18403546	28093/ 5.28	
11	2-cys peroxiredoxin-like protein [*Arabidopsis thaliana*]	31%	334 (>41)	AQADDLPLVGNK APDFEAEAVFDQEFIK LNTEVLGVSVDSVFSHLAWVQTDR SGGLGDLNYPLVSDITK EGVIQHSTINNLGIGR	gi|9758409	29714/ 5.55	0.8 ± 0.04 ↓
12	PSBO-2/PSBO2; oxygen evolving	31%	451 (>42)	RLTYDEIQSK GTGTANQCPTIDGGSETFSFK	gi|15230324	35226/ 5.92	2.92 ± 0.30 ↑

	[*Arabidopsis thaliana*]			FCFEPTSFTVK VPFLFTVK GGSTGYDNAVALPAGGR NTAASVGEITLK SKPETGEVIGVFESLQPSDTDLGAK			
	33 kDa oxygen-evolving protein [*Arabidopsis thaliana*]	31%	430 (>42)	RLTYDEIQSK FCFEPTSFTVK NAPPEFQNTK VPFLFTVK GGSTGYDNAVALPAGGR GDEEELVKENVK NTAASVGEITLK SKPETGEVIGVFESLQPSDTDLGAK	gi|22571	35285/ 5.68	
13	Chlorophyll a/b binding protein (LHCP AB 180) [*Arabidopsis thaliana*]	59%	393 (>41)	GPSGSPWYGSDR YLGPFSGESPSYLTGEFPGDYGWDTAGLS ADPETFAR WAMLGALGCVFPELLAR FGEAVWFK VAGNGPLGEAEDLLYPGGS FDPLGLATDP EAFAELK GPIENLADHLADPVNNNAW AFATNFVPGK	gi|16374	25036/ 5.12	0.29 ± 0.03 ↓
	LHB1B2; chlorophyll binding [*Arabidopsis thaliana*]	24%	259 (>41)	GPSGSPWYGSDR WAMLGALGCVFPELLAR FGEAVWFK GPLENLADHLADPVNNNA WAFATNFVPGK	gi|18403546	28093/ 5.28	
14	Phosphopyruvate	20%	416 (>42)	SAVPSGASTGIYEALELR	gi|15221107	51841/ 5.79	0.41 ±

	hydratase [*Arabidopsis thaliana*]			NQADVDALMLELDGTPNK IGMDVAASEFFMK AAGWGVMVSHR SGETEDNFIADLSVGLASGQIK IEEELGNVR YAGEAFR			0.13 ↓
15	TUA3 [*Arabidopsis thaliana*]	16%	363 (>41)	QLFHPEQLISGK EDAANNFAR SLDIERPTYTNLNR LISQIISSLTTSLR IHFMLSSYAPVISAAK DVNAAVGTIK	gi|15241168	50250/ 4.95	0.66 ± 0.06 ↓
16	ATGRP8 (GLYCINE-RICH PROTEIN 8); RNA binding / nucleic acid binding [*Arabidopsis thaliana*]	44%	515 (>41)	CFVGGLAWATNDEDLQR TFSQFGDVIDSK GFGFVTFK GFGFVTFKDEK VITVNEAQSR SGGGGGYSGGGGGGYSGGGGGGYER	gi|15235002	16626/ 5.58	2.23 ± 0.02 ↑
17	Glycine-rich RNA binding protein 7 [*Arabidopsis thaliana*]	53%	581 (>42)	CFVGGLAWATDDR ALETAFAQYGDVIDSK GFGFVTFK SGGGGGYSGGGGSYGGGGGR EGGGGYGGGEGGGYGGSGGGGGW	gi|21553354	16934/ 5.85	1.73 ± 0.15 ↑
18	AT4g38970/ F19H22_70 [*Arabidopsis thaliana*]	36%	650 (>41)	LDSIGLENTEANR TLLVSAPGLGQYVSGAILFEETLYQSTTEGKK MVDVLVEQNIVPGIK TAAYYQQGAR TVVSIPNGPSALAVK YAAISQDSGLVPIVEPEILLD	gi|16226653	43029/ 6.79	0.65 ± 0.02 ↓

				GEHDIDR ATPEQVAAYTLK ALQNTCLK YTGEGESEEAK			
19	RCA (RUBISCO ACTIVASE) [*Arabidopsis thaliana*]	22%	620 (>42)	LVVHITK VPLILGIWGGK SFQCELVMAK SFQCELVMAK MCCLFINDLDAGAGR IKDEDIVTLVDQFPGQSIDFFGALR LMEYGNMLVMEQENVK VQLAETYLSQAALGDANADAIGR	gi|18405145	52347/ 5.87	2.22 ± 0.37 ↑
20	Glutathione S-transferase (GST6) [*Arabidopsis thaliana*]	24%	288 (>41)	QEAHLALNPFGQIPALEDGDLTLFESR GMFGMTTDPAAVQELEGK VLFDSRPK	gi|20197312	24119/ 6.09	0.55 ± 0.02 ↓
21	Unknown protein [*Arabidopsis thaliana*]	10%	169 (>41)	LSVIVAPVLR FADNLGDDVK IENIGQPAK	gi|2829916	29986/ 6.40	0.44 ± 0.06 ↓
22	Unknown protein [*Arabidopsis thaliana*]	13%	257 (>41)	SAVADNDNGESQVSDVR GKDPIVSGIEDK LSTWTFLPK	gi|6437556	31235/ 5.91	0.67 ± 0.03 ↓
23	ABA-responsive protein [*Pisum sativum*]	48%	487 (>41)	GVFVFDDEYVSTVAPPK LDAVDEANFGYNYSLVGGPGLHESLEK VAFETIILAGSDGGSIVK GDAALSDAVR GDAALSDAVRDETK	gi|20631	16619/ 5.07	Unique
24	ABA-responsive protein [*Pisum sativum*]	39%	250 (>41)	DADEIVPK EAQGVEIIEGNGGPGTIK LSILEDGK	gi|20631	16619/ 5.07	Unique

				VAFETIILAGSDGGSIVK GDAALSDAVR			

[a] Percentage identity between the amino-acids present in MS/MS tag and the sequences in databases.

[b] Ion score is -10 Log (P), where P is the probability that the observed match is a random event. Individual ion scores > value indicate identity or extensive homology ($p<0.05$).

[c] Accession number is Mascot search result using NCBI and other databases.

5.4. Discussion

Abiotic stresses such as salinity, drought, cold and heat negatively affect all stages of plant growth and development. Enhancing plant tolerance to abiotic stresses by introducing genes is a desirable method for many crop plants, but the nature of the genetically complex mechanisms of abiotic stress tolerance, and the potential detrimental side effects of manipulating gene expression, make the task of generating useful transgenic plants very difficult. However, current research efforts to improve plant stress tolerance have had significant success. For example, the over-expression of vacuolar Na^+/H^+ antiporter *AtNHX1* promotes growth and development in saline conditions (up to 200 mM NaCl) in *Arabidopsis* (Apse *et al.*, 1999), and in *B. napus* (Zhang *et al.*, 2001). With respect to tolerance to cold temperatures, transgenic plants over-expressing various transcription factors have been demonstrated to have a higher tolerance. For example, in *Arabidopsis*, significant improvement of freezing stress tolerance was demonstrated by the over-expression of the transcription factor *CBF1* (Jaglo-Ottosen *et al.*, 1998); enhancement of drought and freezing tolerance by overexpression of *CBF4* (Haake *et al.*, 2002) and increased tolerance to freezing, water and salinity stress by over-expression of *DREB1A* gene (Kasuga *et al.*, 1999), whereas transgenic rice over-expressing *CBF3* demonstrated elevated tolerance to drought and salinity but very low freezing tolerance (Oh *et al.*, 2005). It appears, therefore, that these transcription factors are able to enhance tolerance to a variety of stresses. However, such enhancement may be species-specific (Oh *et al.*, 2005). Therefore, it is extremely important to identify other genes/gene products capable of enhancing multiple stress tolerance so as to augment existing tools to engineer stress tolerance. In the extreme cold conditions of the Canadian Prairies, as well as in other parts of the world, successful growth of many crops is based on their ability to germinate and survive better in the cold temperature (Coursolle *et al.*, 1998). Due to risk of early spring or fall frosts, finding novel stress tolerant genes and generation of transgenic plants with enhanced germination and early maturity may have very useful applications for farmers. The results described here suggest that (1) constitutive expression of pea *ABR17* gene in *Arabidopsis* enhances germination and early seedling growth in the presence of salt or cold or when both stresses are combined; and, (2) two week-old transgenic *Arabidopsis* plants exhibit increased tolerance to multiple environmental stresses.

The phytohormone abscisic acid (ABA) plays a very crucial role in response to environmental stresses such as desiccation, salinity and cold and it is also involved in regulating events such as dormancy and maturation during late seed development (McCarty, 1995; Leung and Giraudat, 1998). Many ABA-inducible genes share the *cis*-regulatory elements (i.e. ABA-responsive elements; ABREs; Guiltinan *et al.*, 1990) and have been demonstrated to be involved in the regulation of plant stress responses. Constitutive expression of *ABF3* in *Arabidopsis* (Kang *et al.*, 2002) and rice (Oh *et al.*, 2005) enhances tolerance to drought stress, as well as enhancing tolerance to multiple stresses in *Arabidopsis* (Kim *et al.*, 2004). The pea ABA-responsive *ABR17* gene is induced during late embryogenesis, as well by the exogenous application of ABA (Iturriaga *et al.*, 1994), and the level of the ABR17 protein increases in response to salinity stress (Kav *et al.*, 2004).

Several gene products homologous to ABA-responsive proteins (also known as dehydrins and late embryogenesis abundant or LEA proteins) have been identified in different plant systems (Skriver and Mundy, 1990; Close *et al.*, 1993). It has been suggested that dehydrins and small heat shock proteins (sHSPs) may protect cells from the deleterious effects of dehydration (Pneuli *et al.*, 2002). Expression of: *HVA1* gene of barley, which encodes a LEA protein, confers tolerance to salinity and water-deficit stresses (Xu *et al.*, 1996); the tomato *Le25* gene in yeast increases freezing and salinity tolerance (Imai *et al.*, 1996); and the wheat chloroplast LEA-like protein (WCS19) in *Arabidopsis* (Ndong *et al.*, 2002) increases freezing tolerance. It is possible that ABR17 proteins have a similar function to dehydrins (or LEA) based on the fact that it has a higher content of polar amino acids (about 30%) similar to dehydrins, which also have a higher amount (50%; Pnueli *et al.*, 2002). However, the biological function of ABR17 is currently unknown and, therefore, it is also possible that the mechanism by which ABR17 is able to protect plants is entirely different from that of the dehydrins and must be investigated further.

Despite the identification and characterization of the ABA-responsive protein ABR17, which is similar to the PR 10 proteins from various plant species, its biological function(s) remain unclear. Sequence similarities between an isolated ribonuclease (RNase) from a callus cell culture of *Panax ginseng*, which displays 60–70% sequence identity with two IPRs from parsley, suggests that intracellular PR (IPR) proteins may be RNases (Moiseyev *et al.*, 1994). Other studies have also demonstrated RNase activity of some PR 10 proteins (Bufe *et al.*, 1996; Bantignies *et al.*, 2000; Biesiadka *et al.*, 2002; Wu *et al.*, 2003; Park *et al.*, 2004). Recently, we demonstrated that the pea PR 10.1 protein is a RNase (Srivastava *et al.*, 2006), and because of the similarities between pea PR 10.1 and ABR17 it is possible that pea ABR17 may also be a RNase. We have also demonstrated recently that the constitutive expression of pea *PR 10* cDNA in *B. napus* enhances their germination and early seedling growth (Srivastava *et al.*, 2004).

In order to understand the biological mechanisms underlying ABR17-mediated multiple stress tolerance, we compared the proteome of a transgenic *A. thaliana* line expressing pea *ABR17* gene with its wild type counterpart. As described previously, most of the proteins whose levels were significantly ($P<0.01$) affected in the transgenic line 6.9 are involved in photosynthesis or primary metabolic pathways (Table 5-2). Only those with reported roles in enhancing plant growth and development and/or mediating tolerance to abiotic stresses are discussed further. For example, we observed a significant increase in the levels of PSI-E (spot 2; Table 5-2), a component of photosystem I (PSI). PSI, a multiprotein complex, consists of core proteins and peripheral antenna containing four different light-harvesting Chl a/b protein complexes (LHCs) that mediate the light-driven transfer of electron from plastocyanin to the ferredoxin-NADP complex (Jensen *et al.*, 2003). PSI-E is crucial for growth and development of *A. thaliana* as demonstrated by a 50% reduction in growth as a result of disruption of the *psaE1* gene (Varotto *et al.*, 2000). Therefore, it is possible that an increase in PSI-E protein in our *ABR17* transgenic lines may contribute to the observed increase in stress tolerance.

Another protein that exhibited ~ 3 fold significant up-regulation in a transgenic line is an oxygen-evolving protein, PSBO-2/PSBO2 (spot 12; Table 5-2). The light reaction in oxygenic photosynthesis is catalyzed by the oxygen-evolving PS II and PS I (Steppuhn *et al.*, 1988). Oxygen evolving proteins like PSBO-2/PSBO2, also known as oxygen evolving enhancer (OEE) proteins, and two other molecules form the oxygen-evolving complex function in water splitting and maintenance of an environment conducive for oxygen evolution (James *et al.*, 1989). Levels of OEE are affected by various abiotic stresses including salinity (Abbasi and Komatsu, 2004) and cytokinin treatment (Kasten *et al.*, 1997). Furthermore, OEE from green algae possesses thioredoxin activity (Heide *et al.*, 2004), which can be attributed to well-characterized proteins involved in diverse physiological processes such as amelioration of oxidative stress (Balmer *et al.*, 2004). Therefore, it is possible that OEE2 enhancies the tolerance of *ABR17* transgenic *A. thaliana* by reducing oxidative damage that accompanies abiotic stresses.

In addition to the aforementioned proteins involved in the light reactions of photosynthesis, we also observed a ~2-fold increase in the levels of rubisco activase (RCA), which is involved in the activation of rubisco (Parry *et al.*, 2003). RCA (Spot 19; Figure 5-7; Table 5-2), a member of the AAA$^+$ family, a class of chaperone-like ATPases, activates rubisco by removing sugar phosphates from its active site (Wang and Portis, 1992; Neuwald *et al.*, 1999; Portis, 2003). RCA levels are affected by abiotic stresses, including salinity (Parker *et al.*, 2006) and drought (Salekdeh *et al.*, 2002). It has been suggested that this increase in RCA levels is responsible for mediating tolerance to abiotic stresses by increasing rubisco activity under conditions of low stomatal conductance and CO_2 levels (Parker *et al.*, 2006). It is possible that the transgenic line is able to grow and develop better under stress due to increased photosynthetic efficiency mediated by increased levels of specific PS components and enhanced RCA levels. However, the precise role(s) of these proteins in mediating the observed tolerance must be investigated further and such studies are currently underway in our laboratory.

Another enzyme involved in primary metabolism, phosphopyruvate hydratase (spot 14; Table 5-2), also known as enolase (2-phospho-D-glycerate hydrolase), decreased in the transgenic line. The role of enolase in mediating plant stress responses has been reported previously (Yan *et al.*, 2005 and references therein), and it has been demonstrated that a mutation in *los2* (*low expression of osmotically responsive genes*), which encodes an enolase, enhances the tolerance of *Arabidopsis* to freezing temperature stress (Lee *et al.*, 2002). Therefore, the observed reduction in enolase levels in the *ABR17* transgenic *A. thaliana* line may be important for its tolerance to cold temperature stress and must be investigated further.

Two proteins involved in post-transcriptional regulation, glycine-rich RNA-binding proteins (GR-RBP; spots 16 and 17; Table 5-2), were also observed to be increased in the transgenic *A. thaliana* line used in this study. GR-RBPs contain RNA-recognition motifs at the N-terminus and a glycine rich-region at the C-terminus that are important for their function (Sachetto-Martins *et al.*, 2000; Kim *et al.*, 2005). These proteins have been identified in various species and their involvement in plant development, hormone signaling and stress responses has been suggested (Sachetto-Martins *et al.*, 2000). Although the role of GR-RBPs *in planta* is still not very clear, Kim *et al.* (2005) demonstrated that overexpression of *AtRZ-1a* (a cold-inducible zinc finger-containing GR-RBP) contributes to enhancement of freezing tolerance in

Arabidopsis. Our finding that the levels of two GR-RBPs are enhanced in the transgenic line provides further evidence that this group of proteins may be involved in mediating tolerance to various abiotic stresses.

The results described in this article demonstrated, for the first time, that pea ABR 17 is capable of protecting plants from multiple abiotic stresses, particularly cold and salinity. This protection was evident during the germination of the transgenic seeds in the presence of salt or a combination of salt and cold and also when 2-week-old plants were subjected to freezing stress. The over-expression of pea ABR17 protein also resulted in several interesting protein changes including in photosynthesis-related proteins, enolase and GR-RBPs. Many of these proteins have been previously shown to be associated with enhanced stress tolerance. However, the precise roles of these proteins in mediating the observed tolerance in our transgenic lines remain speculative at this stage. Furthermore, the exact nature of the connections between ABR17 and the proteins that are altered in the transgenic lines needs to be investigated further in order to shed additional light on mechanism(s) underlying ABR17-mediated multiple stress tolerance.

5.5. References

Abbasi, F.M. and Komatsu, S. (2004) A proteomic approach to analyze salt-responsive proteins in rice leaf sheath. Proteomics **4:** 2072–2081.

Agrawal, G.K., Yonekura, M., Iwahashi, Y., Iwahashi, H. and Rakwal, R. (2005) System, trends and perspectives of proteomics in dicot plants. Part III: Unraveling the proteomes influenced by the environment, and at the levels of function and genetic relationships. J Chromatogr B Analyt Technol Biomed Life Sci **815:** 137–145.

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science **285:** 1256–1258.

Balmer, Y., Vensel, W.H., Tanaka, C.K., Hurkman, W.J., Gelhaye, E., Rouhier, N., Jacquot, J.P., Manieri, W., Schurmann, P., Droux, M. and Buchanan, B.B. (2004) Thioredoxin links redox to the regulation of fundamental processes of plant mitochondria. Proc Natl Acad Sci U S A **24:** 2642–2647.

Bantignies, B., Seguin, J., Muzac, I., Dedaldechamp, F., Gulick, P. and Ibrahim, R. (2000). Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. Plant Mol Biol **42:** 871–881.

Biesiadka, J., Bujacz, G., Sikorski, M.M. and Jaskolski, M. (2002) Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. J Mol Biol **319:** 1223–1234.

Borsics, T. and Lados, M. (2002) Dodder infection induces the expression of a pathogenesis-related gene of the family PR-10 in alfalfa. J Exp Bot **53:** 1831–1832.

Boyer, J.S. (1982) Plant productivity and environment. Science **218:** 443–448.

Bray, E.A., Baiely-Serre, J. and Weretilnyk, E. (2000) Responses to abiotic stresses. In: *Biochemistry and Molecular Biology of Plants* (Grusissem, W., Buchannan, B. and Jones, R., eds), pp. 1158–1249. American Society of Plant Physiologists: Rockville, MD.

Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. and Breitenbach., M. (1989) The gene coding for the major birch pollen allergen Betv1, is highly homologous to a pea disease resistance response gene. EMBO J **8:** 1935–1938.

Bufe, A., Spangfort, M.D., Kahlert, H., Schlaak, M. and Becker, W.M. (1996) The major birch pollen allergen, Bet v 1, shows ribonuclease activity. Planta **199:** 413–415.

Chinnusamy, V., Schumaker, K. and Zhu, J.K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot **55:** 225–236.

Close, T.J., Fenton, R.D., Yang, A., Asghar, R., DeMason, D.A., Crone, D.E., Meyer, N.C. and Moonan, F. (1993) Dehydrin: The protein. In: Plant responses to cellular dehydration during environmental stress (Close, T.J. and Bray, E.A., eds), pp. 104–118. Rockville, MD: American Society of Plant Physiologists).

Clough, S. and Bent, A. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743.

Colditz, F., Nyamsuren, O., Niehaus, K., Eubel, H., Braun, H.P. and Krajinski, F. (2004) Proteomic approach: identification of *Medicago truncatula* proteins induced in roots after infection with the pathogenic oomycete *Aphanomyces euteiches*. Plant Mol Biol **55:** 109–120.

Constabel, C.P. and Brisson, N. (1992) The defence-related STH—2 gene product of potato shows race-specific accumulation after inoculation with low concentrations of *Phytophthora infestans* zoospores. Planta **188:** 289–295.

Coursolle, C., Bigras, F.J. and Margolis, H.A. (1998) Frost tolerance and hardening capacity during the germination and early developmental stages of four white spruce (*Picea glauca*) provenances. Can J Bot **76:** 122–129.

Crowell, D.N., John, M.E., Russell, D. and Amasino, R.M. (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol Biol **18:** 459–466.

Duval, F.D., Renard, M., Jaquinod, M., Biou, V., Montrichard, F. and Macherel, D. (2002) Differential expression and functional analysis of three calmodulin isoforms in germinating pea (*Pisum sativum* L.) seeds. Plant J **32:** 481–493.

Fristensky, B., Riggleman, R.C., Wagoner, W. and Hadwiger, L.A. (1985) Gene expression in susceptible and disease resistant interactions of pea induced with *Fusarium solani* pathogens and chitosan. Physiol Plant Pathol **27:** 15–28.

Fristensky, B., Horovitz, D. and Hadwiger, L.A. (1988) cDNA sequence for pea disease resistance response genes. Plant Mol Biol **11:** 713–715.

Goday, A., Jensen, A.B., Culianez-Macia, F.A., Mar Alba, M., Figueras, M., Serratosa, J., Torrent, M. and Pages, M. (1994) The maize abscisic acid-responsive protein Rab17 is located in the nucleus and interacts with nuclear localization signals. Plant Cell **6:** 351–360.

Guiltinan, M.J., Marcotte, W.R. and Quatrano, R.S. (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science **250:** 267–271.

Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F. and Zhang, J.Z. (2002) Transcription factor *CBF4* is a regulator of drought adaptation in *Arabidopsis*. Plant Physiol **130:** 639–648.

Hasegawa, P.M., Bressan, R.A., Zhu, J-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51:** 463–499.

Hashimoto, M., Kisseleva, L., Sawa, S., Furukawa, T., Komatsu, S. and Koshiba, T. (2004) Novel rice PR 10 protein, RSOsPR 10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. Plant Cell Physiol **45:** 550–559.

Heide, H., Kalisz, H.M. and Follmann, H. (2004) The oxygen evolving enhancer protein 1 (OEE) of photosystem II in green algae exhibits thioredoxin activity. J Plant Physiol **161:** 139–149.

Imai, R., Chang, L., Ohta, A., Bray, E.A. and Takagi, M. (1996) A lea-class gene of tomato confers salt and freezing tolerance when expressed in *Saccharomyces cerevisiae*. Gene **170:** 243–248.

Iturriaga, E.A., Leech, M.J., Barratt, D.H.P. and Wang, T.L. (1994) Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. Plant Mol Biol **24:** 235–240.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M.F. (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science **280:** 104–106.

James, H.E., Bartling, D., Musgrove, J.E., Kirwin, P.M., Herrmann, R.G. and Robinson, C. (1989) Transport of proteins into chloroplasts. Import and maturation of precursors to the 33-, 23-, and 16-kDa proteins of the photosynthetic oxygen-evolving complex. J Biol Chem **264:** 19573–19576.

Jensen, P.E., Haldrup, A., Rosgaard, L. and Scheller, H.V. (2003) Molecular dissection of photosystem I in higher plants: topology, structure and function. Physiologia Plantarum **119:** 313–321.

Kang, J.Y., Choi, H.I., Im, M.Y. and Kim, S.Y. (2002) *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343–357.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotech **17:** 287–291.

Kasten, B., Buck, F., Nuske, J. and Reski, R. (1997) Cytokinin affects nuclear- and plastome-encoded energy-converting plastid enzymes. Planta **201:** 261–272.

Kav, N.N.V., Srivastava, S., Goonewardene, L. and Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity. Ann Appl Biol **145:** 217–230.

Kim, J-B., Kang, J-Y. and Kim, S.Y. (2004) Over-expression of a transcription factor regulating ABA-responsive gene expression confers multiple stress tolerance. Plant Biotech J **2:** 459–466.

Kim, Y.O., Kim, J.S. and Kang, H. (2005) Cold-inducible zinc finger-containing glycine-rich RNA-binding protein contributes to the enhancement of freezing tolerance in *Arabidopsis thaliana*. Plant J **42:** 890–900.

Kirk, J.T.O. (1968) Studies on the dependence of chlorophyll synthesis on protein synthesis in *Euglena gracilis*, together with a nomogram for determination of chlorophyll concentration. Planta (Berl.) **78:** 200–207.

Kirk, J.T.O. and Allen, R.L. (1965) Dependence of chloroplast pigment synthesis on protein synthesis: effect of actidione. Biochem Biophys Res Comm **21:** 523–530.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

Lee, H., Guo, Y., Ohta, M., Xiong, L., Stevenson, B. and Zhu, J.K. (2002) LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase. EMBO J **21:** 2692–2702.

Leung, J. and Giraudat, J. (1998) Abscisic acid signal transduction. Annu Rev Plant Biol **49:** 199–222.

McCarty, D.R. (1995) Genetic control and integration of maturation and germination pathways in seed development. Annu Rev Plant Physiol Plant Mol Biol **46:** 71–93.

McGee, J.D., Hamer, J.E. and Hodges, T.K. (2001) Characterization of a PR-10 pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. Mol Plant Microbe Interact **14:** 877–886.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. and Yakovlev, G.I. (1994) High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193:** 470–472.

Ndong, C., Danyluk, J., Wilson, K.E., Pocock, T., Huner, N.P.A. and Sarhan, F. (2002) Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. Plant Physiol **129:** 1368–1381.

Neuwald, A.F., Aravind, L., Spouge, J.L. and Koonin, E.V. (1999) AAA$^+$: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res **9:** 27–43.

Oh, S.J., Song, S.I., Kim, Y.S., Jang, H.J., Kim, S.Y., Kim, M., Kim, Y.K., Nahm, B.H. and Kim, J.K. (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol **138:** 341–351.

Osmark, P., Boyle, B. and Brisson, N. (1998) Sequential and structural homology between intracellular pathogenesis-related proteins and a group of latex proteins. Plant Mol Biol **38:** 1243–1246.

Park C.J., Kim K.J., Shin R., Park J.M., Shin Y.C. and Paek K.H. (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J **37:** 186–198.

Parker, R., Flowers, T.J., Moore, A.L. and Harpham, N.V. (2006) An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. J Exp Bot **57:** 1109–1118.

Parry, M.A., Andralojc, P.J., Mitchell, R.A., Madgwick, P.J. and Keys, A.J. Manipulation of Rubisco: the amount, activity, function and regulation. (2003) J Exp Bot **54:** 1321–1333.

Pnueli, L., Hallak-Herr, E., Rozenberg, M., Cohen, M., Goloubinoff, P., Kaplan, A. and Mittler, R. (2002) Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume *Retama raetam*. Plant J **31:** 319–330.

Portis, A.R.Jr. (2003) Rubisco activase - Rubisco's catalytic chaperone. *Photosynth Res* **75:** 11–27.

Ramonell, K.M. and Somerville, S. (2002) The genomics parade of defense responses: to infinity and beyond. Curr Opin Plant Biol **5:** 291–294.

Sachetto-Martins, G., Franco, L.O. and de Oliveira D.E. (2000) Plant glycine-rich proteins: a family or just proteins with a common motif? Biochim Biophys Acta **1492:** 1–14.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. and Bennett, J. (2002) Proteomic analysis of rice leaves during drought stress and recovery. Proteomics **2:** 1131–1145.

Schardl, C., Byrd, A., Benzion, G., Altschuler, M., Hildebrand, D. and Hunt, A. (1987) Design and construction of a versatile system for the expression of foreign genes in plants. Gene **61:** 1–11.

Skriver, K. and Mundy, J. (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512.

Somssich, I.E., Schmelzer, E., Kawalleck, P. and Hahlbrock, K. (1988) Gene structure and in situ transcript localization of pathogenesis-related protein 1 in parsley. Mol Gen Genet **213:** 93–98.

Srivastava, S., Fristensky, B. and Kav, N.N.V. (2004) Constitutive expression of a PR 10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol **45:** 1320–1324.

Srivastava, S., Emery, R.J.N., Kurepin, L.V., Reid, D.M., Fristensky, B. and Kav, N.N.V. (2006) Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels. Plant Growth Regul **49:** 17–25.

Steppuhn, J., Hermans, J., Nechushtai, R., Ljungberg, Ulf., Thummler, F., Lottspeich, F. and Herrmann, R.G. (1998) Photosynthesis is driven by two photosystems PSI and PSII in higher plants. FEBS Letters **237:** 218–224.

Subramanian, B., Bansal, V.K. and Kav, N.N.V. (2005) Proteome-level investigation of *Brassica carinata*-derived resistance to *Leptosphaeria maculans*. J Agric Food Chem **53:** 313–324.

Van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Reporter **12:** 245–264.

Van Loon, L.C. and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol **55:** 85–97.

Varotto, C., Pesaresi, P., Meurer, J., Oelmuller, R., Steiner-Lange, S., Salamini, F. and Leister, D. (2000) Disruption of the *Arabidopsis* photosystem I gene psaE1 affects photosynthesis and impairs growth. Plant J **22:** 115–124.

Vettakkorumakankav, N.N., Falk, D., Saxena, P. and Fletcher, R.A. (1999) A crucial role for gibberellins in stress protection of plants. Plant Cell Physiol **40:** 542–548.

Walter, M.H., Liu, J.W., Grand, C., Lamb, C.J. and Hess, D. (1990) Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of proteins including pollen allergens. Mol Gen Genet **222:** 353–360.

Wang, W., Vinocur, B. and Altman, A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218:** 1–14.

Wang, Z.Y. and Portis, A.R. Jr. (1992) Dissociation of Ribulose-1,5-Bisphosphate bound to Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase and its enhancement by Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase activase-mediated hydrolysis of ATP. Plant Physiol **99:** 1348–1353.

Wu, F., Li, Y.K., Chang, S., Zhou, Z., Wang, F., Song, X., Lin, Y. and Gong, W. (2003) Purification, characterization and preliminary crystallographic studies of a PR-10 protein from *Pachyrrhizus erosus* seeds. Acta Crystal D **58:** 2165–2167.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T-H.D. and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol **110:** 249–257.

Yan, S., Tang, Z., Su, W. and Sun, W. (2005) Proteomic analysis of salt stress-responsive proteins in rice root. Proteomics **5:** 235–244.

Zhang, H.X., Hodson, J.N., Williams, J.P. and Blumwald, E. (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc Natl Acad Sci USA **98:** 12832–12836.

Zhu, J-K (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol **6:** 441–445.

6. A crucial role for cytokinins in pea *ABR17*-mediated enhanced germination and early seedling growth of *Arabidopsis thaliana* under saline and low temperature stresses

6.1. Introduction

Plants respond to pathogenic infections and abiotic stress conditions by inducing the expression of a number of genes that encode pathogenesis-related (PR) proteins. The term pathogenesis-related (PR) proteins was originally proposed by Antinov *et al.* (1980) to describe proteins grouped into 14 different families based on their amino-acid sequence, serological relationship and/or enzymatic or biological activities (van Loon *et al.*, 1994; van Loon and van Strien, 1999). Most of the PR proteins, with the notable exception of the intracellular pathogenesis-related (IPR) proteins of the PR 10 class, are secreted (van Loon *et al.*, 1994). PR 10 proteins are ubiquitous in the plant kingdom and represent a class of soluble acidic proteins of molecular mass between 15 and 18 kDa (van Loon and van Strien, 1999).

PR 10 proteins are encoded by multigene families and are resistant to proteases (Warner *et al.*, 1994) and have been reported to be expressed under the biotic and abiotic stress conditions (Srivastava *et al.*, 2006^a and references therein).

A version of this chapter is communicated and under review: ***Srivastava S.****, Rahman H., Emery R.J.N. and Kav N.N.V. (2006) A crucial role for cytokinins in pea ABR17-mediated enhanced germination and early seedling growth of* Arabidopsis thaliana *under saline and low temperature stresses* ***Journal of Plant Growth Regulation*** *(in press).*

Some PR 10 proteins are developmentally regulated and are constitutively expressed in different plant organs in other plant species (Crowell *et al.*, 1992; Barratt and Clark, 1993; Sikorski *et al.*, 1999). These species-dependent and developmentally-regulated variations in expression patterns suggest their possible involvement, not only in plant defence mechanisms, but also in general plant development (Wu *et al.*, 2002). Although gene expression patterns under various stress conditions, accumulation in various plant organs, and physicochemical properties of PR 10 are well characterized, the precise biological functions of PR 10 proteins in plant growth and development are unknown. Based on high amino-acid sequence similarities between PR 10 proteins and ribonucleases, it has been suggested that PR 10 proteins possess RNase activity (Moiseyev *et al.*, 1994). Furthermore, PR 10 members from *Betula spp.* Bet v 1 (Bufe *et al.*, 1996), white lupin (Bantignies *et al.*, 2000), *Pachyrrhizus erosus* (Wu *et al.*, 2002), hot pepper (Park *et al.*, 2004), pea (Srivastava *et al.*, 2006[a]) and a few other species have been reported to possess RNase activity.

PR 10 proteins also bind many types of molecules including cytokinins, brassinosteroids, fatty acids and flavonoids, leading to the suggestion that all PR 10 proteins may not be RNases (Fujimoto *et al.*, 1998; Mogensen *et al.*, 2002; Markovic-Housely *et al.*, 2003). Based on weak sequence homology and secondary-structure prediction, the cytokinin specific-binding proteins (CSBPs) have also been included in the PR 10 class (Fujimoto *et al.*, 1998). Structural studies by Mogensen *et al.* (2002) and Markovic-Housley *et al.* (2003) have also suggested that PR 10 proteins function as a carrier for hormone during the plant defense response to pathogens, as well as during normal growth and developmental processes (Pasternak *et al.*, 2005). Thus, despite our increased knowledge of *PR 10* expression, the biological function of these proteins is currently unknown.

In peas, PR 10 proteins are encoded by a family of genes comprised of five known members: *PR 10.1-10.3*; also known as *Drr49a-c*; and pea ABA-responsive proteins (*ABR*) *17* and *18,* also known as *PR 10.4* and *10.5*, respectively (Tewari *et al.*, 2003). Pea *ABR17* and *18* are expressed during seed development and are, as the name implies, induced by exogenous application of ABA (Iturriaga *et al.*, 1994; Colditz *et al.*, 2004). Reports in the literature described increases in levels of PR 10 proteins in response to stresses; furthermore, we demonstrated previously that the levels of pea PR 10.1, as well as ABR17 proteins are increased in the roots of salinity-stressed pea plants (Kav *et al.*, 2004). These observations suggested that PR 10.1 and ABR17 have roles in protecting plants from the deleterious effects of salinity stress, a hypothesis that is supported by the demonstration that the constitutive expression of pea *PR 10.1* cDNA in *Brassica napus* enhances its germination and early seedling growth under saline conditions (Srivastava *et al.*, 2004). We also demonstrated that pea PR 10.1 possesses RNase activity and also that the concentrations of cytokinins (CKs) are significantly elevated in those transgenic *B. napus* seedlings overexpressing Pea *PR 10.1* cDNA (Srivastava *et al.*, 2006[a]). Salinity influences the levels of pea ABR17 in response to salinity stress in pea roots (Kav *et al.*, 2004), suggesting that this protein has a similar protective effect. This hypothesis was tested using the model plant *Arabidopsis thaliana*, which confirmed that *ABR17* overexpression enhances germination and early seedling growth under saline, as well as low temperature stress conditions (Srivastava *et al.*, 2006[b]).

Due to the similarities in the stress-protective effects that were observed as a result of the constitutive expression of both pea *ABR17* and *PR 10.1* in two different plant species, we wanted to investigate whether pea *ABR17* was mediating its effects through CKs. In this paper we tested whether exogenous CK application can simulate the protective effects of *ABR17* expression in wild type *A. thaliana*, particularly within the context of enhanced germination and seedling growth. We determined the relative levels of *ABR17* and a primary CK-responsive gene, and the endogenous concentrations of CKs in three independently derived *ABR17* transgenic *A. thaliana* plants. Furthermore, the RNase activity of purified recombinant ABR17 was tested.

6.2. Materials and methods

6.2.1. Plant material and growth conditions

Transformation of *A. thaliana* with the pea *ABR17* cDNA and the generation of three transgenic lines (line 6.9, 14.9 and 25.20) have been previously described (Srivastava *et al.*, 2006^b). Seeds from transgenic, homozygous plants of the T_2 generation and the wild type (ecotype WS) were germinated and grown in plastic trays containing Metro Mix® 290 (Grace Horticultural products, Ajax, Ontario) in the greenhouse (22°C day/18°C night, 16 h photoperiod) supplemented with Sylvania 400 watt high pressure sodium (HPS) lamps, 940 μmol m^{-2} s^{-1} and fertilized with a complete fertilizer (Peters™20-20-20, Plant Products, Bramalea, Ontario) containing micronutrients. The plants were allowed to grow to seed maturity and phenological observations were made. In addition, tissue from 2-week-old plants grown in green house was also collected for phytohormone analysis.

Wild type and transgenic lines (6.9, 14.9, 25.20) of *A. thaliana,* ecotype WS seeds were also grown on ½ strength MS medium containing 1.5% sucrose, 0.8% agar, pH 5.7 in 25 x 100 mm Petri dishes and at least five plates per treatment and 14 seeds per plates were used in these experiments. The plates were incubated at 22±1°C under continuous fluroscent light 30 μmol m^{-2} s^{-1} and sterile tissue from 2-week-old plants was collected for RNA and phytohormone analysis. Seeds were rinsed for one min with 70% ethanol, surface sterilized with 20% bleach for 15 min after which they were washed four times (5 min each) with sterile deionized water to remove the bleach. The sterilized seeds were placed on solid ½ strength MS medium containing 1.5% sucrose, 0.8% agar, pH 5.7, for 2 weeks after which the seedlings were harvested.

6.2.2. Exogenous application of CKs

Two different approaches were used to test the ability of various CKs to enhance germination and early seedling growth of wild type *A. thaliana* under conditions of stress. In one experiment, wild type *A. thaliana* seeds were surface sterilized as described previously and placed on medium containing various concentrations (1, 5 and 25 μM) of CKs (mixed isomer-zeatin, *trans*-zeatin riboside, BAP, 2-iP or kinetin) with or without 150 mM NaCl in 25 x 100 mm petri dishes. In the second approach, surface sterilized wild type seeds were imbibed in 25 μM aqueous solutions of different CKs [mixed isomer-Zeatin, *trans*-zeatin riboside, 6-Benzyl amino purine (BAP), 6-(γ,γ- Dimethyl allyl amino) purine (2iP) or kinetin] for 4 h, washed 4 times (5 min each) with sterile DW, after which they were placed on plates containing 150 mM NaCl but without CK. The plates containing seeds were then incubated at 21±1°C under continuous fluroscent light 30 μmol m^{-2} s^{-1} or at 10±1°C in a growth chamber (Conviron, model PGR15, Winnipeg, Manitoba) with coolwhite supplemented with incandescent bulbs 250 μmol m^{-2} s^{-1}. Both types of experiments were designed in a randomized complete block design with at least five plates per treatment (14 seeds per plate), and seedlings were grown for two weeks. Each experiment was repeated at least three times and the percentage of germinated seeds calculated for each and the data statistically analyzed as described below.

6.2.3. Quantitative real time (RT)-PCR

Quantitative RT-PCR was performed using TaqMan® system, to investigate the relative levels of expression of the *ABR17* and *Arabidopsis* response regulator (*AtARR*) 5, and expression of actin was used as the control. PCR primer sets for RT-PCR were designed using the Primer Premier 3 software (Applied Biosystems Inc., CA, USA) to generate PCR products of approximately 70-80 bp. The primer sets used in these experiments for the various genes are shown in Table 6-1.

Table 6-1. Primer sequences for over expression and quantitative real-time PCR analysis

Gene **(Access. No.)**	**Primer pairs and probe used in real time PCR**
PsABR17 (gi|20630)	Forward; 5'- AAATGGAGGTCCAGGAACCAT-3'
	Reverse; 5'- AGCACATAGTTGGTTTTTCCATCTT-3'
	Probe; 5'- AGAAGCTATCCATTCTT-3'
AtActin (gi|20465864)	Forward; 5'-GCCATTCAGGCCGTTCTTT-3'
	Reverse; 5'-ATCGAGCACAATACCGGTTGT-3'
	Probe; 5'- TCTATGCCAGTGGTCG-3'
AtARR5 (gi|3953598)	Forward; 5'- TTCATTAGCATCACCGAAACTTCT-3'
	Reverse; 5'- CGATGAACTTCCGATCAACCA-3'
	Probe; 5'- TTCTTGCTGTTGATGATAG-3'

Total RNA was extracted using the QIAGEN RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) from 2-week-old seedling tissue and treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA. First strand cDNA was synthesized by reverse transcription of total RNA (50 ng) using the iScript cDNA synthesis kit (Bio-Rad, CA, USA). The PCR reaction (20 μL) contained 2 μl of 5 X diluted cDNA as template, 22.5 pmol of each primer, 5 pmol probe and 1x TaqMan UniversPCR Master Mix (Roche, New Jersey, USA). The real time quantification was performed in an ABI prism 7700 Sequence detector (Applied Biosystems) using the SNP RT template program. The comparative relative expression was determined using the delta-delta method employing the formula relative expression = $2^{-[\Delta\mathrm{Ct\ sample} - \Delta\mathrm{Ct\ control}]}$ (Livak and Schmittgen, 2001), where Ct refers to the threshold cycle. Level of expression in the wild type was considered to be 1 and the relative expression in the transgenic lines was normalized against these levels. The reactions were performed in duplicate and the experiments were repeated at least three times.

6.2.4. Determination of endogenous CK concentrations

Endogenous concentrations of the various phytohormones in 2-week-old *A. thaliana* seedlings (wild type and the transgenic lines) were grown in the greenhouse as well as on solid ½ strength MS medium as described earlier (Srivastava *et al.*, 2006[a]). Tissue harvested from either greenhouse grown plants or aseptic cultures was flash frozen in liquid nitrogen, lyophilized and used for phytohormone analysis. CKs were extracted, purified and quantified by an isotope dilution assay under conditions established by Emery *et al.* (1998, 2000) and Ferguson *et al* (2005). Deuterated CKs, $[^2H_6]$iP, $[^2H_6][9R]$iP, *trans*-$[^2H_5]$Z, $[^2H_3]$DZ, *trans*-$[^2H_5][9R]$Z, $[^2H_3][9R]$DHZ, $[^2H_6][9R\text{-}MP]$iP and $[^2H_6][9R\text{-}MP]$DHZ (OlChemIm Ltd, Olomouc, Czech Republic) were added as quantitative internal standards. Nucleotides were converted to nucleosides for quantification. Purified CK were separated and analyzed by (LC-(+)ESI-MS/MS) using a Waters 2680 Alliance HPLC system (Waters, Milford, USA) linked to a Quattro-LC triple quadrupole MS (Micromass, Altrincham, UK).

6

6.2.5. Over expression and purification of pea ABR17 in *E. coli*

The overexpression and purification of pea ABR17 was performed using the methods previously described for the purification of recombinant pea PR 10.1 (Srivastava *et al.*, 2006[a]). Pea *ABR17* cDNA was amplified using two specific primers: Forward 5'-GTG GTC GCA TAT G**GA AAA TTT GTA CTT TCA AGG T**AT GGG TGT CTT TGT TTT TGA TGA TGA ATA C-3' and Reverse 5'-TAT ATA GCT CGA GTT AGT AAC CAG GAT TTG CCA AAA CGT AAC C-3'. The forward primer possessed an Nde I (underlined), as well as the rTEV protease recognition sequence (bold), whereas the reverse primer contained an Xho I recognition site (underlined). PCR-amplified products were digested with Nde I and Xho I (New England Biolabs, ON, Canada) and ligated into similarly digested pET28a bacterial expression vector (Novagen, WI, USA) in order to generate a hexahistidine-tagged fusion protein upon expression. *E. coli* expression cells (Rosetta DE3;

Novagen) were transformed with the ligation mix and colonies containing the recombinant construct were identified and the sequence of *ABR17* verified by sequence analysis. *E. coli* cells harboring the recombinant pET28a plasmid containing the *ABR17* open reading frame were cultured in Luria-Bertani liquid media containing kanamycin (50 µg/mL) with agitation (250 rpm) at 37°C to an absorbance of 0.4-0.8 at 600 nm. The bacterial culture was then cooled to room temperature (23°C) and IPTG (1mM final concentration) was added to induce PR 10.4 expression. Induction proceeded at room temperature with shaking for 3 h, after which the culture was centrifuged (at 7,000 rpm, 10 min, 4°C).

The bacterial pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, 1% Triton X100 and complete (EDTA-free) protease inhibitor cocktail – Roche Inc., USA) pH 7.5. Bacterial cells were lysed using a SONIC 300 Dismembrator (Artek Systems Corp., NY, USA) with 10 cycles of 30 sec each at a setting of 0.8 relative output. Between each cycle a 1-min cooling step on ice was included. After lysis, the bacterial cell suspension was centrifuged at 10,000 rpm for 10 min at 4°C, the supernatant collected and applied to a Ni-NTA agarose column (Qiagen) that was previously equilibrated with lysis buffer. The flow through was collected and reapplied to the column once more in order to ensure the complete adsorption of the expressed recombinant ABR17. Bacterial proteins that were weakly adsorbed were removed by washing first with 20 column volumes of wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM β-mercaptoethanol, 1% Triton X100, 10% glycerol, 15 mM imidazole, complete EDTA-free protease inhibitor cocktail, pH 7.5) and then with 15 column volumes of wash buffer 2 (similar to buffer 1 but with 30 mM imidazole). Recombinant ABR17 protein was eluted from the column by applying 5 column volumes of wash buffer 3 (buffer 1 with 150 mM imidazole). The eluted recombinant ABR17 protein was dialyzed overnight against rTEV protease buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT) and cleaved with the rTEV protease (Invitrogen, CA, USA; 1.2 U/µg) by incubation at 30ºC for 5 h followed by an overnight incubation at 4ºC. The cleaved ABR17 protein was purified by passing it through a Ni-NTA agarose column and collecting the flow through.

6.2.6. RNase activity measurements

RNase activity of the purified, recombinant ABR17 was determined using an in-solution, as well as an in-gel assay as previously described for the pea PR 10.1 protein (Srivastava *et al.*, 2006[a]). Briefly, for the in-solution activity assays, the reaction mixtures contained 6 µg total pea or canola RNA and 0, 3, 6 or 9 µg uncleaved or cleaved proteins in rTEV buffer (50 mM Tris-HCl, pH 8, 0.5 mM EDTA and 1 mM DTT). After incubation for 3 h at RT, an equal volume of phenol-chloroform was added to the reaction mixture and the reaction mixture was vortexed. The reaction mixture was centrifuged at 14,000 rpm for 2 min and the upper layer (aqueous phase) was placed in a fresh microfuge tube and analyzed on a 1.2% agarose gel. For the in-gel activity assay, the recombinant ABR17 proteins were separated on a 15% acrylamide gel containing 2.4 mg/mL yeast tRNA using a Mini PROTEAN 3 vertical slab system (Bio-Rad). Following electrophoresis, the SDS was removed and the proteins renatured by washing the gels twice in a solution of 25% isopropanol (v/v) in 0.01 M Tris-HCl for 10 min each at RT and then washed twice with 0.01 M Tris-HCl for 10 min each at RT. The gel was incubated in 0.1 M Tris-HCl at 51°C for 50 min and stained with 0.2% (w/v) toluidine blue O (Aldrich Inc., USA) in 0.01 M Tris-HCl for 10 min at RT. The gel was destained by incubating twice with 0.01 M Tris-HCl for 10 min each after which it was rinsed and stored in 10 % glycerol-0.01 M Tris-HCl.

6.2.7. Statistical analysis

The experiment was designed in a randomized complete block design (RCBD). All statistical analyses were performed using the mixed model procedure of SAS version 8e (Statistical Analysis System, 1985). Two way analysis of variance (ANOVA) was performed using a mixed model and least square means were separated using a Pdiff option for significant ($P<0.05$) fixed effects. An estimate of least square means and pooled standard errors are presented in graphs.

6.3. Results

6.3.1. Appearance of *ABR17* transgenic *A. thaliana* plants

The appearance of wild type and three independently derived transgenic lines (lines 6.9, 14.9 and 25.20) at various stages of growth and development are shown in Figure 6-1 (A-E). It is clear from the images presented in Figure 6-1 that the transgenic lines were developmentally further ahead of the wild type at all stages. Both the wild type and transgenic lines started to bolt during third week, but bolting was earlier in all transgenic lines, in particular in line 6.9, as compared to the wild type counterpart (Figure 6-1C). Days-to-opening of the first flower was also earlier (at least 24-48 h) in transgenic lines than the the wild type.

The number of lateral branches originating from the primary inflorescence was significantly greater ($P<0.01$) in the transgenic lines. The number of siliques in each plant was counted when the stem turned brown (~ 6 weeks) and the number of mature siliques was found to be higher in the transgenic lines. The transgenic line 6.9 possessed a significantly ($P<0.05$) higher number of siliques as compared to the other two transgenic or the wild type line (Figure 6-2B). These results indicate that introduction of pea *ABR17* cDNA had positive effects on enhancing growth and development of transgenic *A. thaliana* plants.

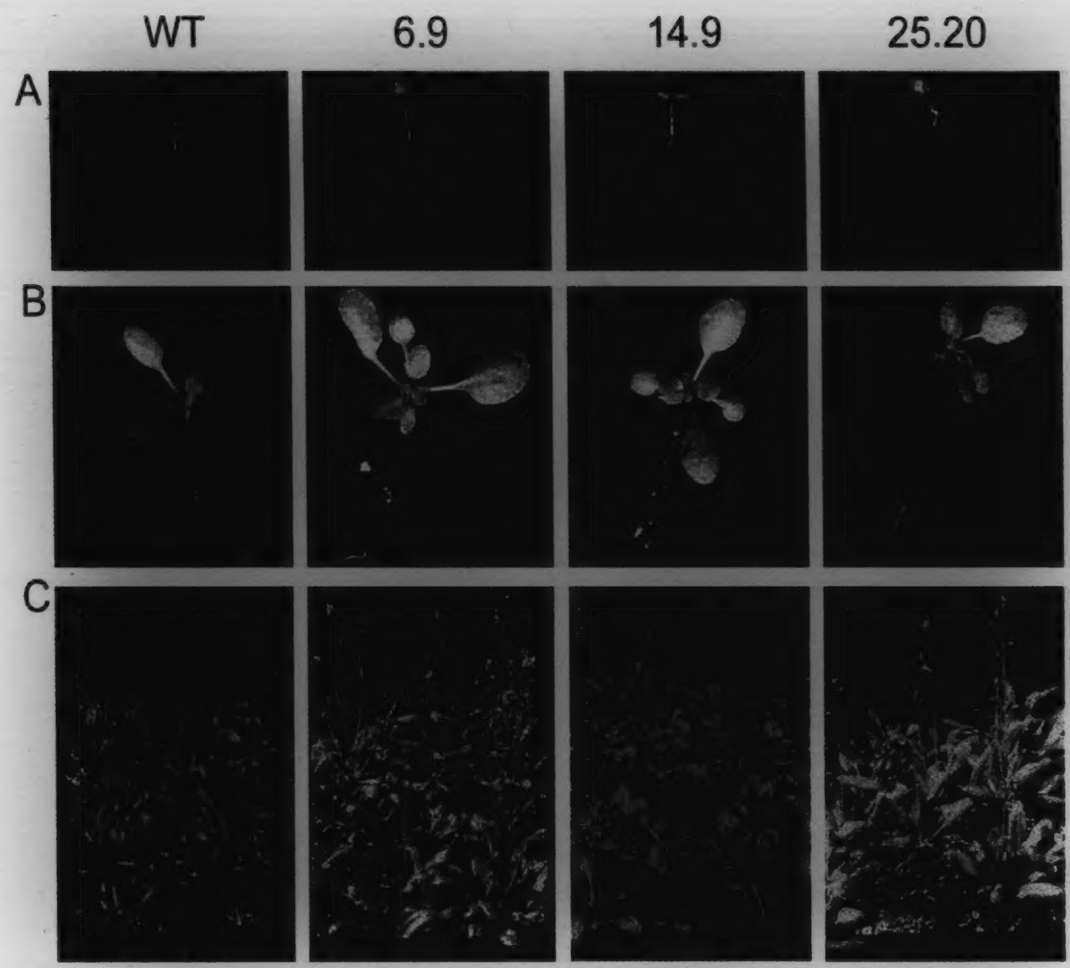


Figure 6-1. Enhanced germination and overall growth of wild type and transgenic (6.9, 14.9 and 25.20) *A. thaliana* constitutively expressing pea *ABR17* cDNA. Appearance after (A) 5 days (B) 2 weeks (C) 3 weeks.

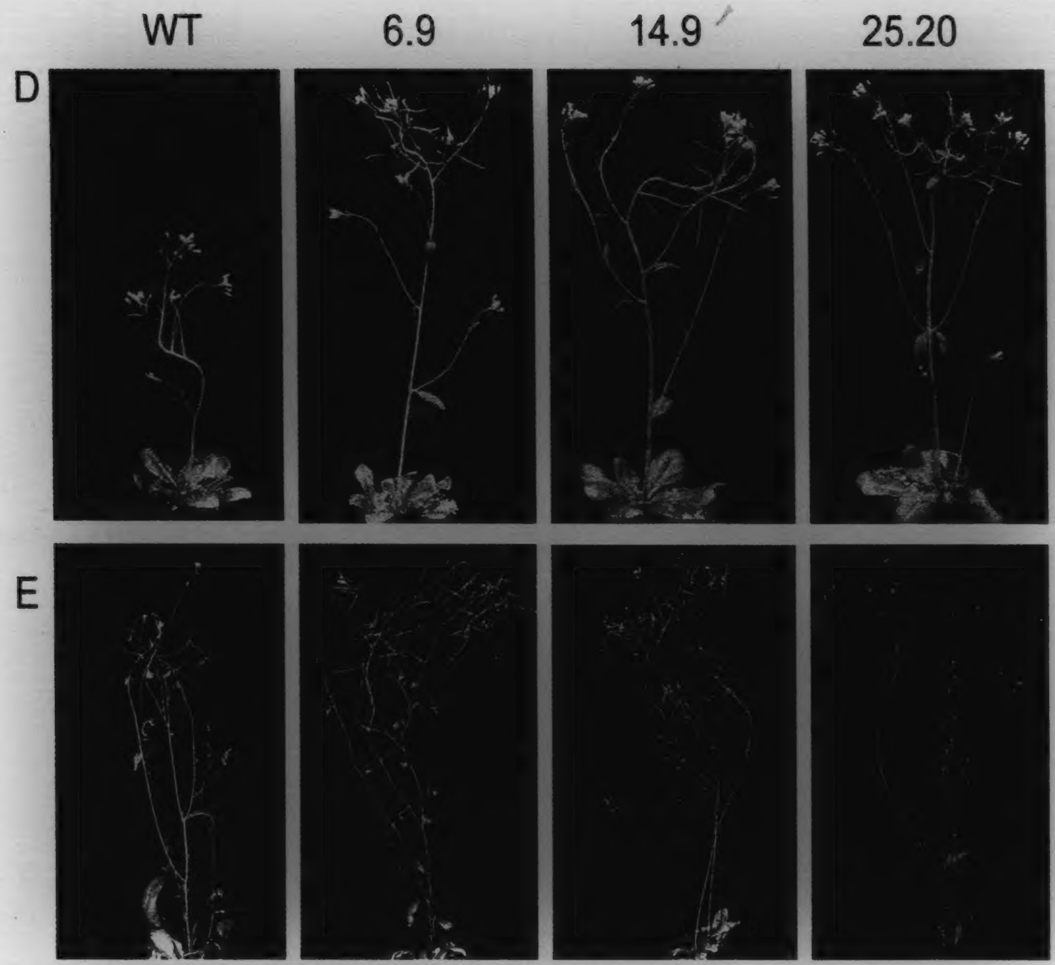


Figure 6-1 (contd). Enhanced germination and overall growth of wild type and transgenic (6.9, 14.9 and 25.20) *A. thaliana* constitutively expressing pea *ABR17* cDNA. Appearance after (D) 4 weeks and (E) 5 weeks.

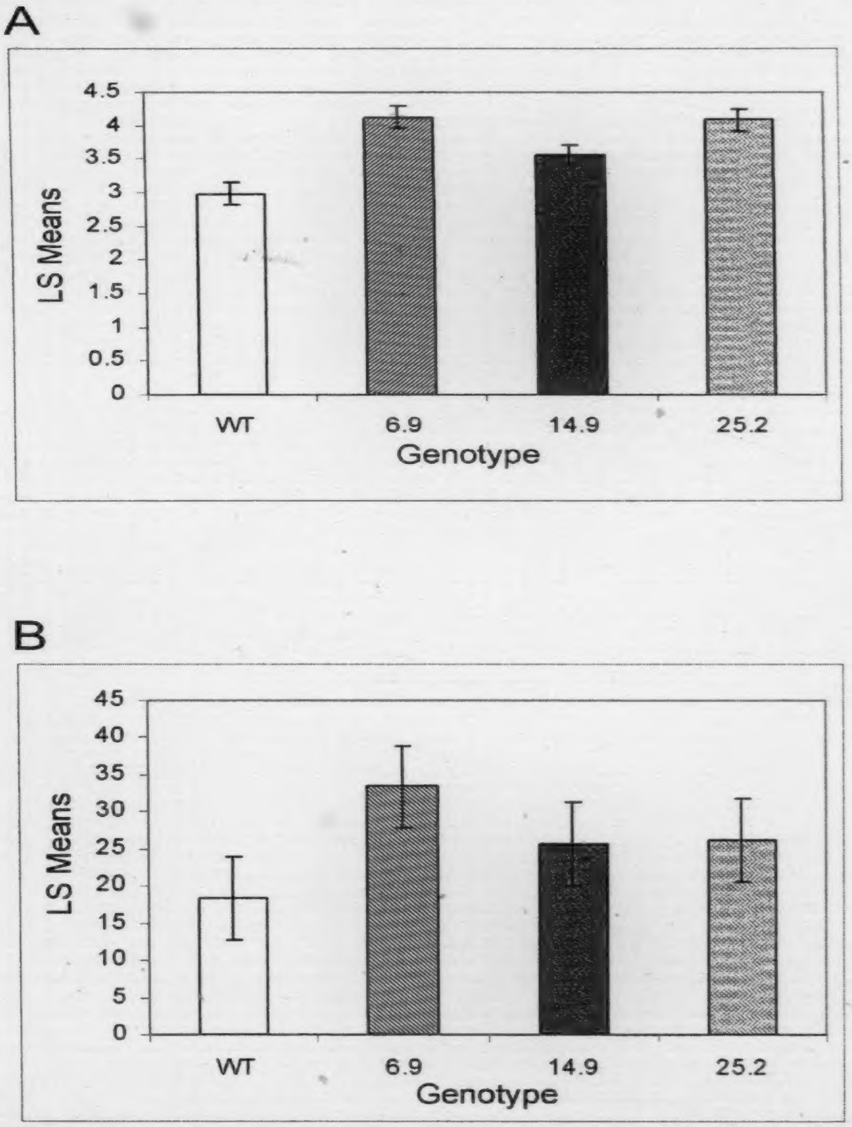


Figure 6-2. Increased lateral branching and siliques in *ABR17*-transgenic *A. thaliana*. (A) Lateral branches and (B) siliques in wild type and three *ABR17* transgenic lines (6.9, 14.9 and 25.20). An estimate of least square means and pooled standard errors are presented.

6.3.2. Effects of exogenous CK on germination of *A. thaliana* under salinity and cold stress

As mentioned previously, the two transgenic lines (6.9 and 25.20) exhibit enhanced germination and seedling growth under abiotic stress conditions (Srivastava *et al.*, 2006[2]). *ABR17* is considered to be one of the members of the pea PR 10 protein family and the constitutive expression of another pea PR 10 protein (PR 10.1) has been demonstrated to enhance germination of *B. napus* (Srivastava *et al.*, 2004) as well as endogenous CK levels (Srivastava *et al.*, 2006[a]). Based on these studies we hypothesized that the enhanced germination of *ABR17* transgenic *A. thaliana* under abiotic stress may be mediated by CK. In order to test this possibility, we performed experiments where we tested the ability of various CKs to enhance germination and subsequent development of wild type *A. thaliana* seedlings under those conditions using two different approaches. In the first approach, where various CKs were included in the culture medium at a concentration of 5 μM, the percentage of seeds germinating in the presence of 150 mM NaCl at RT was significantly ($P<0.05$) higher on day 5 and remained higher till the conclusion of the 14-day experiment in the presence of CKs compared to control plates with no exogenous CK (Figure 6-3A).

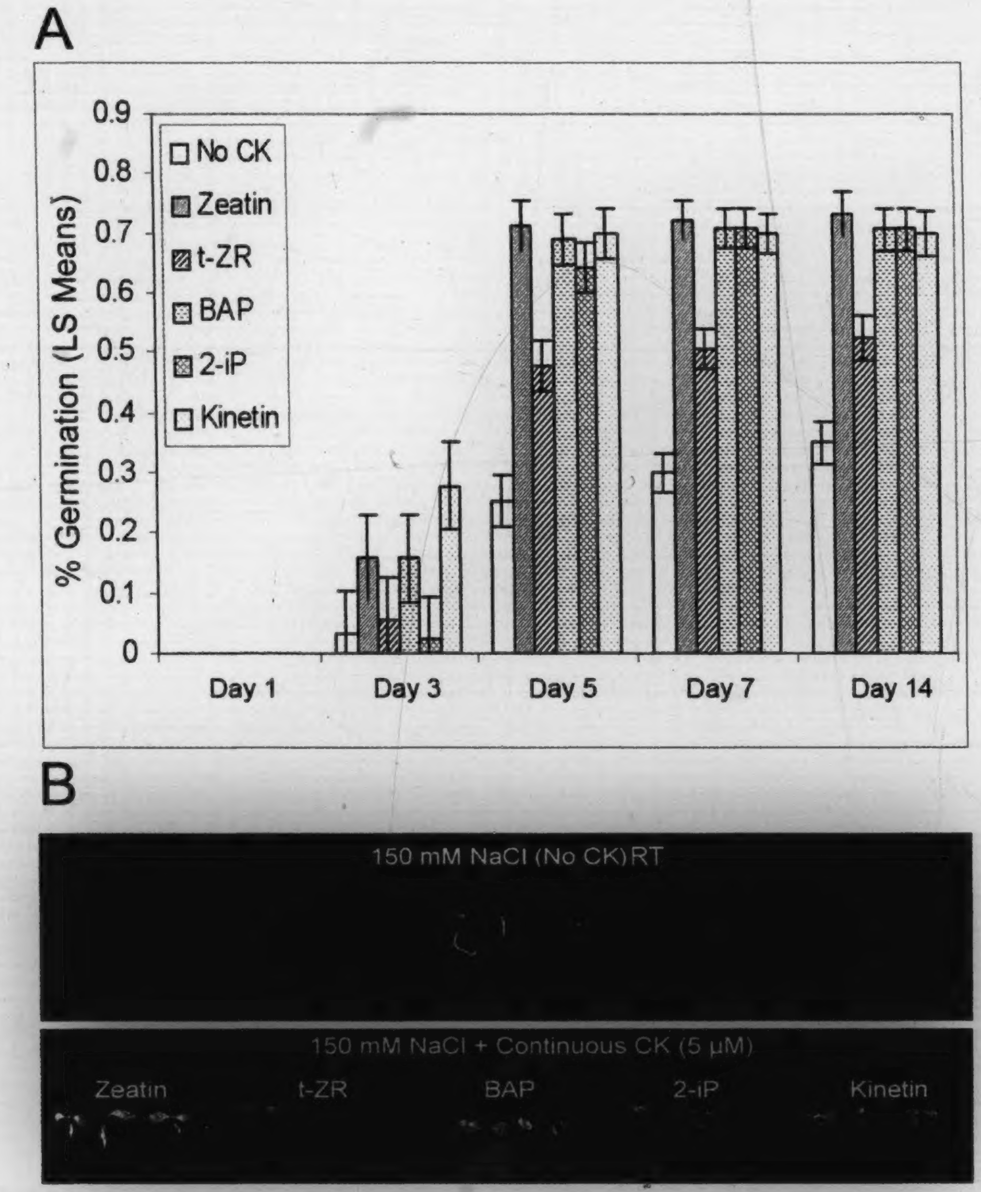


Figure 6-3. Effects of CK on the wild type *A. thaliana* when included in the MS medium. (A) Germination and (B) appearance of wild type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at RT. An estimate of least square means and pooled standard errors are presented.

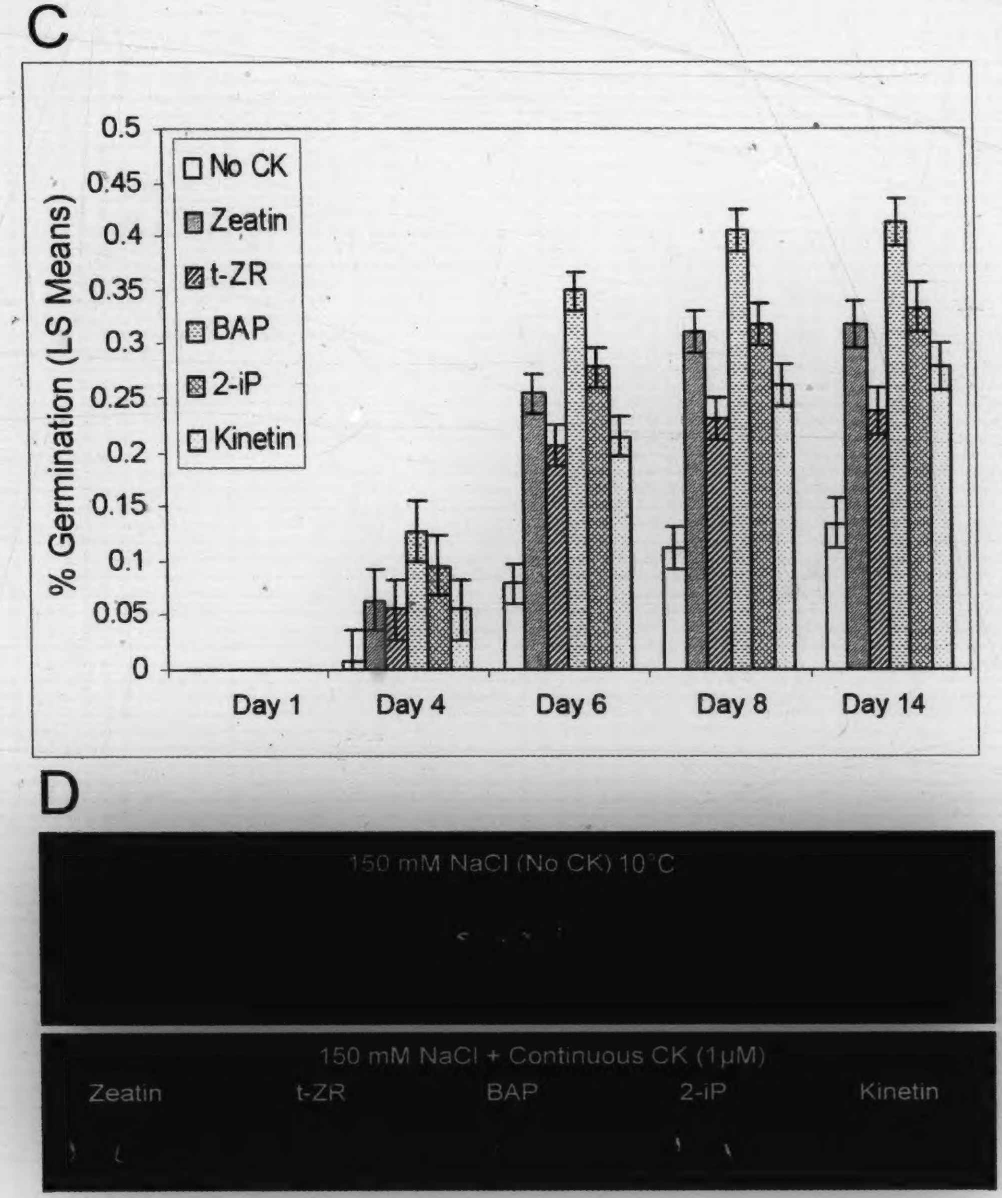


Figure 6-3 (contd.). Effects of CK on the wild type *A. thaliana* when included in the MS medium (C) germination and (D) appearance of wild type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at 10ºC. An estimate of least square means and pooled standard errors are presented.

The appearance of the seedlings after the 14-day experiment for this treatment is shown in Figure 6-3B and it is evident that the treated seedlings were developmentally more advanced than untreated seedlings. At the end of the 14-day period, the CKs zeatin, benzylamino purine (BAP), isopentenyl adenine (2 iP) and kinetin enhanced the germination of *A. thaliana* in the presence of 150 mM NaCl, significantly (Figure 6-3A). Even though *trans*-ZR was able to enhance germination under these conditions, the extent of this enhancement was lower than the other CKs tested (Figure 6-3A).

The ability of CKs to enhance germination in the presence of 150 mM NaCl at 10°C was also evaluated and the results are shown in Figures 6-3C-D. Preliminary experiments at this temperature showed that 1 μM CK concentration was the most effective in enhancing the germination when included in the growth medium (data not shown). In the presence of 1 μM concentration of various CKs and 150 mM NaCl at this lower temperature, the percentage of seeds that germinated were significantly ($P<0.05$) higher than in the absence of CKs and this enhancement was apparent on day 6 and continued until day 14 (Figure 6-3C). All types of CKs tested were equally effective with perhaps BAP being the most effective and *trans*-ZR being the least when observed at the conclusion of the 14-day experiment (Figure 6-3D). The appearance of the seedlings at the conclusion of the experiment (Figure 6-3D) also supported the above conclusions.

In the second approach, when wild type seeds were imbibed in 25 μM aqueous solutions of different CKs and subsequently placed on ½ strength MS plates in the presence of 150 mM NaCl at RT, a significant increase in the percentage of germinated seeds was observed even on day 3 and remained high until the conclusion of the experiment on day 14 (Figure 6-4A). Thus both approaches demonstrated a significant enhancement in germination by exogenous application of various CKs. The notable difference between the two types of approaches to administer the CKs was that in the first approach zeatin was most effective, (Figure 6-3A) and in the second approach, 2 iP was most effective (Figure 6-4A), and in both the approaches the *trans*-ZR was least effective. Once again, as can be seen from the appearance of the seedlings at the conclusion of the 14-day experiment, those that were treated with CKs were more advanced than untreated controls (Figure 6-4B).

When the seeds were imbibed in CK solutions as described earlier and placed on plates containing 150 mM NaCl at 10ºC, germination of the treated seeds was enhanced (Figures 6-4C and D). This enhancement was evident even on day 4 and continued until the end of the 14-day experiment at which time all the CKs tested enhanced germination significantly ($P<0.05$). Similar to what we observed in this type of CK treatment at RT, *trans*-ZR was the least effective in enhancing the percentage of germinated seeds at 10ºC as well (Figures 6-4C and D).

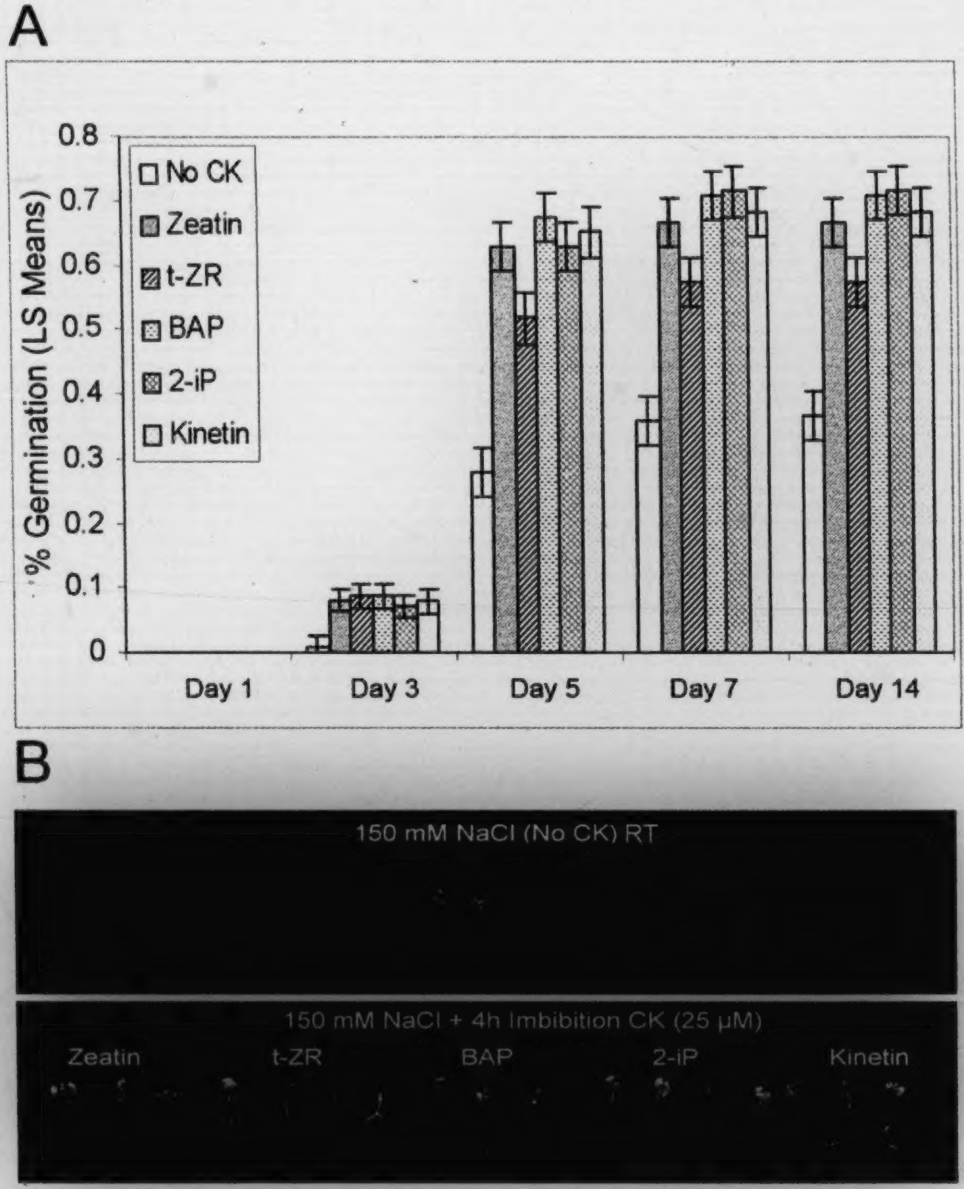


Figure 6-4. Effects of CKs when wild type *A. thaliana* seeds imbibed in aqueous CK solutions. (A) Germination and (B) appearance of wild type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at RT. An estimate of least square means and pooled standard errors are presented.

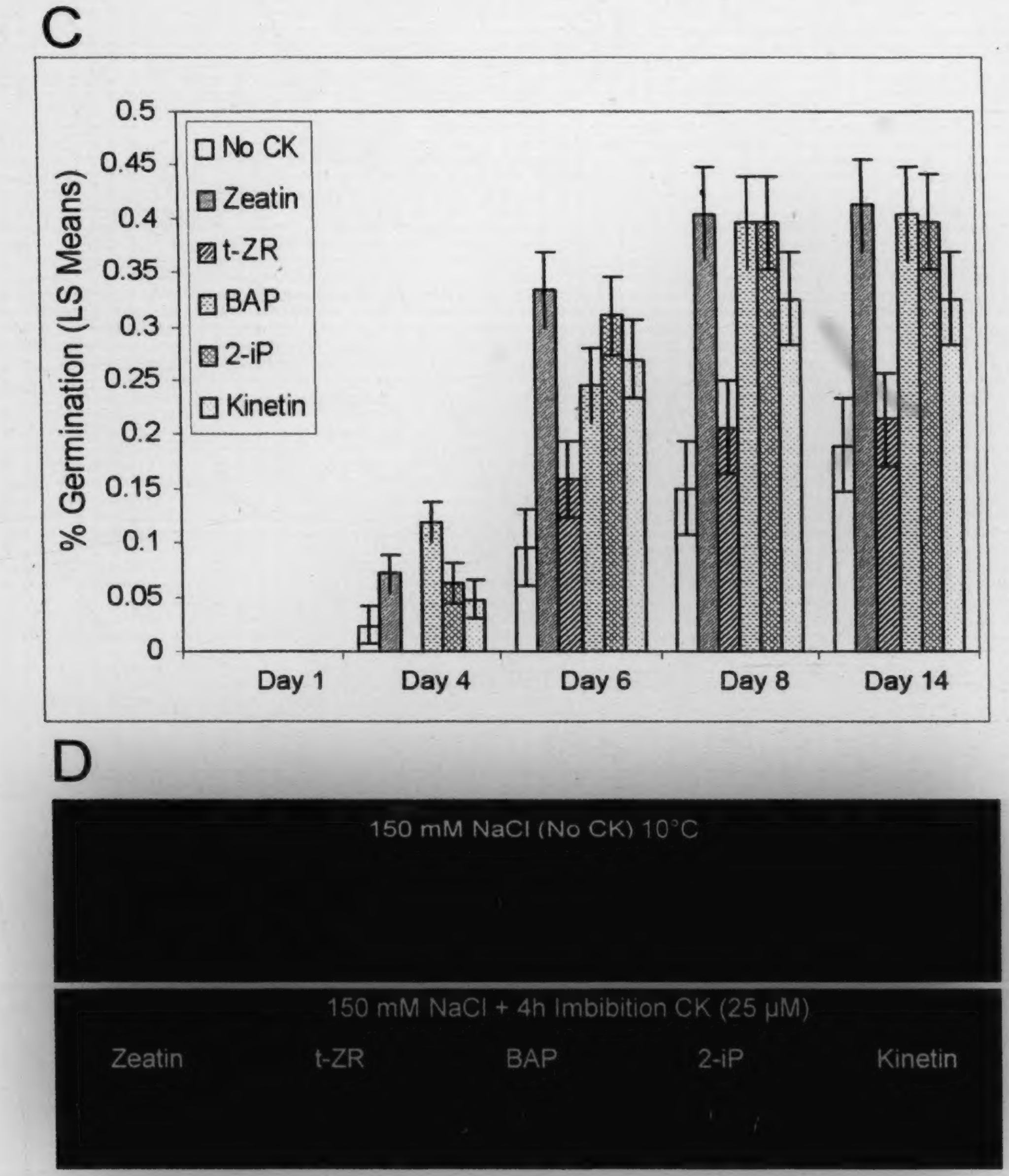


Figure 6-4 (contd). Effects of CKs when wild type *A. thaliana* seeds imbibed in aqueous CK solutions. (C) germination and (D) appearance of wild type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at 10ºC. An estimate of least square means and pooled standard errors are presented.

6.3.3. Analysis of *ABR17* and *AtARR5* gene expression

The relative levels of expression of the pea *ABR17* gene in the three transgenic lines (6.9, 14.9 and 25.20) were assessed using quantitative RT-PCR. In addition, to further test our hypothesis that the stress protective effects of *ABR17* expression are mediated, at least in part, due to an increase in CKs, we also investigated the relative expression of the CK primary response gene *AtARR5* using Q-RT-PCR. Among the transgenic lines, 6.9 had the highest level of *ABR17* expression and the other two transgenic lines had comparable levels (Table 6-2). The levels of expression of the primary CK response regulator *AtARR5* also correlated well with that of *ABR17* with all transgenic lines showing increased levels of the *AtARR5* transcript (Table 6-2). Furthermore, the level of *AtARR5* expression was also highest in line 6.9, which also correlates with this line exhibiting the highest level of *AtABR17* expression (Table 6-2). The other two transgenic lines had approximately half the level of *AtARR5* expression compared to line 6.9.

Table 6-2. Gene expression analysis (relative fold expression)

Gene	**Relative fold expression***			
	WT	**6.9**	**14.9**	**25.20**
PsABR17	1	3115 ± 1143	961 ± 278	1128 ± 301
AtARR5	1	9 ± 1	4.3 ± 0.5	4.1 ± 0.2

* Gene expression in the wild type was considered to be 1 and the relative expression in the transgenic lines was normalized against the wild type expression level.

6.3.4. Endogenous concentration of CKs in *ABR17* transgenic *A. thaliana*

The increase in *AtARR5* transcript levels in the three transgenic lines suggested a link between the expression of pea *ABR17* and *AtARR5*. Since *ARR5* is thought to be the primary CK responsive gene, the increase in *AtARR5* expression in *ABR17* transgenics further strengthens our hypothesis that *ABR17* may be mediating its effects through CKs. This is also supported by the enhanced germination of wild type *A. thaliana* under abiotic stress conditions in the presence of exogenous CKs as previously discussed. In order to further investigate the role of CKs in the *ABR17*-mediated effects, we determined the endogenous levels of CKs in the three transgenic as well as wild type *A. thaliana* seedlings.

The endogenous levels of various types of CKs, their nucleoside and nucleotide precursors are shown in Table 6-3. In 2-week-old seedling tissue obtained from MS plates, two of the three transgenic lines (6.9 and 25.20) has higher concentratins of various endogenous CKs (i.e. *cis*-Z, *trans*-Z, *cis*-[9RMP]Z and *trans*-[9RMP]Z), whereas the concentrations of these CKs were not different in transgenic line 14.9 when compared to the wild type (Table 6-3). A dramatic increase in the levels of *trans*-ZR and a moderate increase in [9R]iP were observed in line 25.20 (tissue from MS plate), but not in the other two transgenic lines (Table 6-3). In addition, the concentrations of [9R]iP in tissues of MS-grown transgenic lines 6.9 and 25.20 were also higher than wild type seedlings (Table 6-3).

Table 6-3. Endogenous concentrations of CK in wild type and transgenic *A. thaliana*

Cytokinins (CK)	**Quantity (pmol gDW^{-1})**							
	Wild-type (Mean ± SE)		**6.9 (Mean ± SE)**		**14.9 (Mean ± SE)**		**25.20 (Mean ± SE)**	
	MS plates grown plants (n = 2)	Soil grown plants (n = 3)	MS plates grown plants (n = 2)	Soil grown plants (n = 3)	MS plates grown plants (n = 2)	Soil grown plants (n = 3)	MS plates grown plants (n = 2)	Soil grown plants (n = 3)
trans-Z	25.1 ± 3.3	46.7 ± 0.8	40.6 ± 7.0	48.2 ± 0.8	28.0 ± 0.1	41.1 ± 2.0	87.2 ± 0.3	44.2 ± 1.6
cis-Z	66.5 ± 1.3	23.6 ± 0.9	87.0 ± 4.4	42.6 ± 0.9	69.8 ± 1.4	43.7 ± 1.0	105.8 ± 2.7	46.8 ± 1.8
iP	2.6 ± 0.1	2.0 ± 0.1	5.9 ± 1.2	5.2 ± 0.8	2.2 ± 0.1	2.5 ± 0.0	38.5 ± 0.6	3.1 ± 0.1
trans-[9R]Z	92.5 ± 1.3	268.9 ± 1.9	88.3 ± 7.0	213.3 ± 1.2	63.5 ± 0.3	207.9 ± 1.6	262.2 ± 0.5	151.4 ± 5.2
cis-[9R]Z	11.5 ± 0.8	15.1 ± 0.3	9.8 ± 0.7	18.3 ± 0.9	8.9 ± 0.3	11.0 ± 0.6	14.7 ± 1.5	12.6 ± 0.5
[9R]DHZ	0.0 ± 0.0	20.6 ± 0.6	0.0 ± 0.0	20.1 ± 0.4	0.0 ± 0.0	22.1 ± 0.5	22.8 ± 0.1	20.8 ± 2.1
[9R]iP	54.7 ± 1.3	95.5 ± 0.6	36.6 ± 2.1	95.6 ± 0.5	38.0 ± 0.4	79.6 ± 1.7	65.2 ± 2.2	86.5 ± 1.5
FB total	252.9 ± 0.3	472.4 ± 3.0	268.2 ± 21.1	443.3 ± 1.4	210.4 ± 2.4	408.0 ± 4.7	596.3 ± 1.5	365.3 ± 2.9
trans-[9RMP]Z	203.5 ± 24.7	1060.5 ± 27.5	315.8 ± 0.8	1058 ± 13.2	209.3 ± 1.9	938.5 ± 22.1	460.8 ± 10.8	715.7 ± 72.5
cis-[9RMP]Z	49.8 ± 11.4	56.9 ± 1.19	75.5 ± 13.4	82.1 ± 2.9	56 ± 1.1	41.7 ± 2.2	93.2 ± 3.5	36.7 ± 4.2
[9RMP]DHZ	19 ± 2.8	37.3 ± 1.09	21.4 ± 8	30.8 ± 2.8	25.6 ± 2.6	36.6 ± 0.9	28.4 ± 4.3	34.2 ± 0.6
[9R]iP	273 ± 1.3	518.8 ± 6.5	342.7 ± 5.6	561.9 ± 11.1	234.2 ± 0.4	482.9 ± 0.3	298 ± 4.7	597.9 ± 9.8
NT total	545.3 ± 17.5	1673.5 ± 32.5	755.4 ± 11.7	1732.9 ± 22.5	525 ± 1.4	1500 ± 24	880.4 ± 7	1384.4 ± 83.7
Total CK	798.2 ± 17.8	2145.8 ± 35.5	1023.6 ± 32.7	2176.1 ± 21.3	735.5 ± 3.7	1907.7 ± 28.7	1476.7 ± 8.5	1749.7 ± 80.5

In 2-week-old soil grown plants, that the concentration of *cis*-Z was higher in all three transgenic lines, whereas only line 25.20 demonstrated an increase in concentration of *cis*-[9RMP]Z (Table 6-3). The concentrations of *trans*-Z did not change among four genotypes, whereas the *trans*-ZR and *trans*-[9RMP]Z showed a decrease in all three transgenic lines, which contributed towards the overall reduction in the concentrations of free CK bases in all the transgenic lines and a reduction in concentration of CK nucleotides in lines 14.9 and 25.20 (Table 6-3). We should emphasize that CKs were measured only at a single developmental stage (immediately prior to bolting) and this may not have captured all the differences between the three transgenic lines and the wild type. However, it is clear that in tissue from MS-grown *A. thaliana* seedlings, significant increases in specific CK forms are found in the transgenic lines.

6.3.5. RNase activity of pea ABR17 protein

PR 10 proteins isolated from different plant species may or may not possess RNase activity (Wu *et al.*, 2003 and references therein; Srivastava *et al.*, 2006[a]). We have previously reported that the pea PR 10.1 protein is a RNase (Srivastava *et al.*, 2006[a]). In order to determine whether pea ABR17, which also possesses many of the amino acid residues implicated in the RNase activity, is also an RNase, we overexpressed the cDNA in *E. coli* and purified the recombinant protein using metal-chelation affinity chromatography as previously described for pea PR 10.1 (Srivastava *et al.*, 2006[a]).

The recombinant protein was expressed at significant levels upon induction with IPTG and we were successful in purifying the protein with or without the histidine tag (Figure 6-5A). Both forms of the protein (with or without the histidine tag) were tested for RNase activity using in-solution and in-gel activity assays (Srivastava *et al*., 2006[a]). It is clear from the results shown in Figure 6-5B that both forms of the protein were able to degrade RNA isolated from pea and canola. As described earlier, an in-gel assay was also performed to investigate whether the recombinant ABR17 protein is an RNase (Figure 6-5C). Proteins with RNase activities upon separation in this gel system containing yeast tRNA produced clear regions at the expected molecular weights (Srivastava *et al*., 2006[a]). As can be observed from the gel shown in Figure 6-5C, we observed a clear region against a blue background precisely at the molecular weight of the recombinant protein with and without the histidine tag. The identity of the protein was also verified as pea ABR17 by excising the band from an SDS-PAGE performed under identical conditions but without the yeast tRNA and tandem Mass Spectrometry (data not shown).

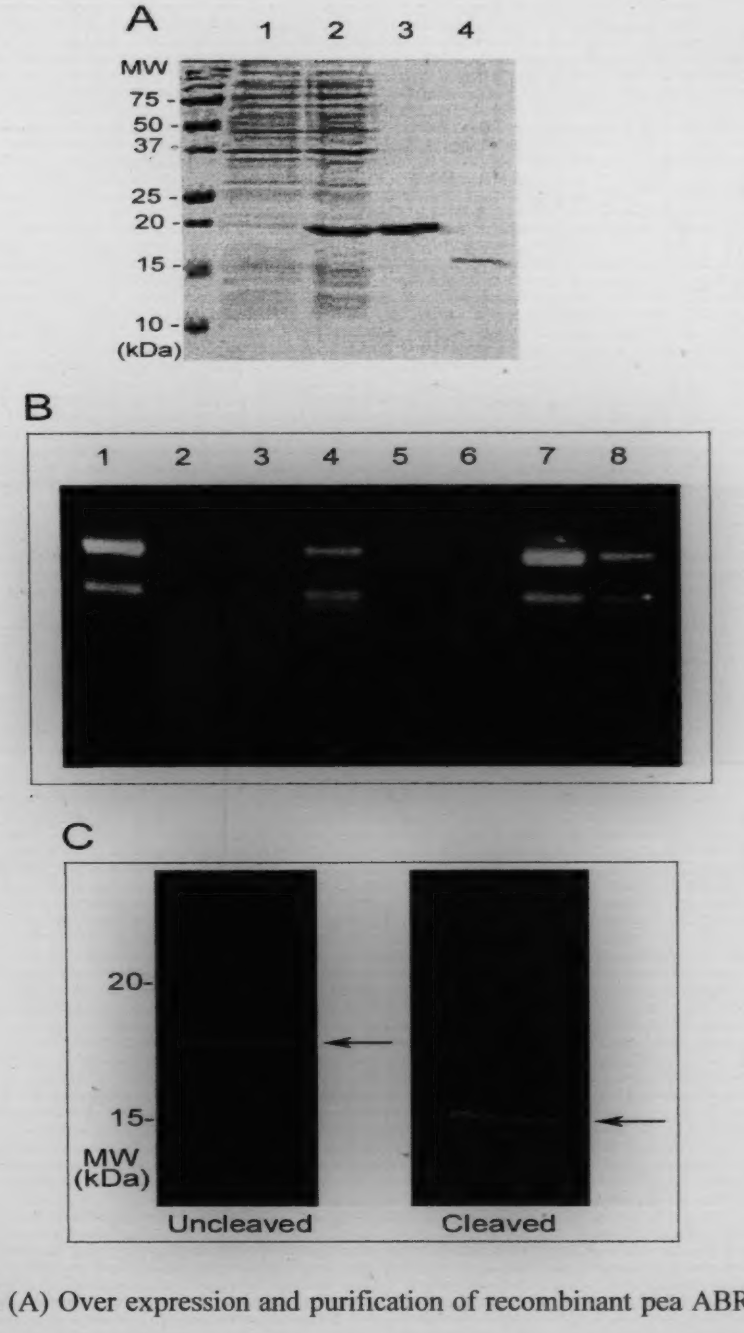

A
1
2
3
4
MW
75 -
50 -
37 -
25 -
20 -
15 -
10 -
(kDa)
B
1
2
3
4
5
6
7
8
C
20-
15-
MW
(kDa)
Uncleaved
Cleaved

Figure 6-5. (A) Over expression and purification of recombinant pea ABR17. Lanes 1 and 2 represent uninduced and induced *E. coli* cultures, respectively and lanes 3 and 4 represent purified recombinant ABR17 protein (Continued on next page) with or without the histidine tag, respectively. (B) In-solution RNA degradation assay with recombinant ABR17. Lanes 1-3 and 7 are reactions with total RNA from pea, and lanes 4-6 and 8 with total RNA from canola. Lanes 2, 5 are reactions with 9 µg recombinant ABR17 protein with the histidine tag and lanes 3, 6 are reactions with 6 µg recombinant ABR17 protein without the histidine tag. Lane 7 is total RNA from pea incubated with 9 µg recombinant ABR17 protein with the histidine tag which had been boiled in a boiling water bath for 15 min and lane 8 is total RNA from canola incubated with recombinant 6 µg ABR17 protein without the histidine tag which had also been boiled for 15 min. (C) In-gel RNase activity. Arrows point to clear bands that are produced as a result of RNase activity and correspond to the molecular weights of recombinant ABR17 with or without the histidine tag.

6.4. Discussion

In this study, we investigated mechanisms underlying previously reported pea ABR17-mediated enhancement of germination of *A. thaliana* under saline and low temperature stress conditions. Results from our previous study indicated that the constitutive expression of a pea *PR 10* cDNA (*PR 10.1*) increases endogenous concentrations of CKs, suggesting an important role for CKs in ameliorating the deleterious effects of salinity stress (Srivastava *et al.*, 2006[a]). Since pea ABR17 is considered to be a member of the pea PR 10 protein family, we hypothesized that the observed stress tolerance during germination may be mediated by a modulation of endogenous CKs. Thus we performed an experiment where the effects of exogenous CKs on the germination of wild type *A. thaliana* were tested. Our results indicated that almost all the CKs tested were able to promote germination under saline and low temperature stresses suggesting an important role for CKs in mediating this phenomenon.

Our next experiment was to investigate the levels of *ABR17* transgene expression in three independently-derived (Srivastava *et al.*, 2006[b]) transgenic *A. thaliana* lines (6.9, 14.9 and 25. 20), as well as the expression of the primary CK-responsive gene, *ARR5* (Hwang and Sheen, 2001). Our results indicated that all transgenic lines had greater *ABR17* expression than controls and that this was most pronounced in line 6.9. Lines 14.9 and 25.20 were not significantly different with respect to *ABR17* expression (Table 2). Similarly, we observed a 4-9 fold increase in the levels of *ARR5* transcript in the transgenic lines with the highest increase (~9 fold) in line 6.9, which further suggests a direct relationship between *ABR17* and *ARR5* expression in our transgenic *A. thaliana* lines.

Even though our exogenous CK application, as well as Q-RT-PCR analysis of gene expression, suggested a role for CKs in enhancing germination, and a direct relationship between *ABR17* and *ARR5* expression, additional evidence for *ABR17*-mediated modulation of CKs in the transgenic lines is necessary to further substantiate our hypothesis. In order to achieve this objective, we determined the endogenous concentrations of various forms of CKs using LC-MS/MS in tissue obtained from seedlings grown on MS plates as well as in soil. Tissue from 2-week-old seedlings grown on MS plates were used because our earlier study revealed that transgenic seedlings grown in this manner are significantly more tolerant to abiotic stresses (Srivastava *et al.*, 2006^b). Tissue from soil-grown plants was also used for endogenous CK determinations because these plants exhibited differences in their phenotypes (Figure 6-1) when compared to the wild type. Some of these differences included precocious flowering, a higher degree of lateral branching and increased number of siliques (Figure 6-2), all of which are traits that have been connected to an increases in CK activity (Bonhomme *et al.*, 2000; Mader *et al.*, 2003; Liu *et al.*, 2004; Riefler *et al.*, 2006; Tanaka *et al.*, 2006). Our results indicated that the total endogenous concentrations of CK in tissues of 2-week-old transgenic lines 6.9 and 25.20 grown on MS plates increased, whereas the total concentrations in tissues from soil-grown plants generally decreased (Table 6-3). The MS plate results provide additional, direct evidence that the expression of pea *ABR17* in *A. thaliana* modulates CK levels as previously hypothesized.

Our results also indicated that levels of specific forms of CKs are being consistently altered in the transgenic lines. For example, in tissues from plants grown in both conditions, an increase in the concentration of *cis*-Z was observed. Similarly, the endogenous concentration of *cis*-[9RMP]Z also increased in the transgenic lines 6.9 and 25.20 when grown in MS plates, whereas in the soil-grown plants an increase in *cis*-[9RMP]Z was observed only in line 6.9 (Table 6-3). The endogenous concentration of *trans*-Z, a form of CK that is considered to be biologically active (Yamada *et al.*, 2001), also increased in tissue of transgenic lines 6.9 and 25.20 obtained from MS-grown seedlings. However, the tissue from soil-grown seedlings did not show any differences in the concentration of this form of CK (Table 6-3). Another CK that showed a consistent increase in both the systems is iP, also considered to be a biologically active form, where the concentrations increased in the two transgenic lines (6.9 and 25.20) when grown on MS plates.

The observed differences in the endogenous concentrations of specific forms of CKs in the transgenic lines correlated well with the ability of certain CKs to enhance the germination of wild type *A. thaliana*. For example, zeatin (mixed isomers) supplied continuously for 2 weeks in the growth media resulted in enhanced germination in the presence of NaCl, as well as under low temperature stress (Figure 6-3D), which correlated well with an increase in the endogenous concentration of both *cis*-Z and *trans*-Z in the transgenic lines 6.9 and 25.20. Similarly, the imbibition of seeds in aqueous solutions of iP prior to germination under these stresses, enhanced germination in the presence of both types of abiotic stresses investigated in this study. These results also agreed well with our observation that the endogenous concentration of iP was also elevated in the two transgenic lines 6.9 and 25.20. Interestingly, the endogenous CK concentrations did not increase in line 14.9 which also did not exhibit the enhanced germination and stress tolerance observed with the other two lines (6.9 and 25.20; Srivastava *et al.*, 2006[b]). Furthermore, the levels of *ABR17* and *ARR5* were similar in both lines 25.20 and 14.9, which may be due to positional effects and/or other uncharacterized differences between the two lines, which may include developmental differences between these lines. However, what is consistent is the fact that CKs increased in two out of the three transgenic lines tested and, this increase correlates with the observed stress tolerance.

A small number of PR 10 proteins including the pea PR 10.1 possess RNase activity. However, the role of RNase activity in the *in planta* function of these proteins is not clear (Srivastava *et al.*, 2006[a]). In order to test whether pea ABR17 is a RNase, we expressed the cDNA in *E. coli* and purified the hexahistidine-tagged protein to homogeneity and assayed the purified recombinant protein for RNase activity. Our results (Figure 6-5) indicated that recombinant pea ABR17 (with or without the histidine tag) possessed RNase activity which is similar to that of pea PR 10.1, even though the degree of similarity at the amino acid level is ~58% between pea ABR17 and 10.1. Together with an increase in endogenous CK in the *ABR17*-transgenic *A. thaliana* lines, these results suggest the possibility that the RNase activity of ABR17 is responsible for the increase in endogenous CKs as previously suggested for pea PR 10.1 protein (Srivastava *et al.*, 2006[a]).

The likelihood that the RNase activity of ABR17 contributes to increased concentrations of CK is supported by existence of two CK biosynthesis pathways in plants: one involves the mevalonate (MVA) pathway, located in the cytosol, that prenylates tRNA and is mainly responsible for producing *cis*-Z derivatives; and, another localized in plastids involves the methylerythritol phosphate (MEP) pathway and provides the prenyl precursor to *trans*-CK and iP-type CK (Kasahara *et al.*, 2004; Sakakibara, 2006). tRNAs have been suggested to be a possible source of endogenous CKs because of isopentenylation of an adenine residue in these molecules (Swaminathan *et al.*, 1977) and it has been estimated that in plants tRNAs contribute as much as 40–50% of cytokinin pools (Barnes *et al.*, 1980; Letham and Palni, 1983; Prinsen *et al.*, 1997), but others have argued that the slow turnover of tRNAs indicates that tRNA molecules do not contribute significantly to endogenous CK pools (Barnes *et al.*, 1980; Klambt, 1992). However, it is difficult to determine the rate of tRNA turnover and CK production accurately and therefore, it is not possible to properly assess the contributions of tRNA degradation as a source of free, active, CKs (Miyawaki *et al.*, 2004). It has also been suggested that tRNA degradation is a source of *cis*-Z type CKs (Sakakibara, 2006) and iP is the basic moiety in tRNA reported in all biological kingdoms, including plant tRNAs albeit as a minor source of CK (Prinsen *et al.*, 1997). The consistent increase in the levels of *cis*[9R-MP]Z, *cis*-Z and, to a lesser extent, iP in two of the three transgenic lines (6.9 and 25.20) suggest the possibility that increased synthesis of these CK species may occur by degradation of tRNA. Based on the results of Kasahara *et al* (2004), this is the type of CK profile that would be predicted in the event of increased tRNA degradation. Moreover, since the relative activities of the *cis*- versus *trans*-CK, and cytosol- versus plastid-localized CK are unknown, focus should be placed on changes in the the *cis*-CK levels, even though the *trans*-CK levels were higher overall. Our results are also typical of *A. thaliana* found by Kasahara *et al* (2004), whereby the trend in amount of individual CKs was as follows: nucleotide CK > ribosyl-CKs > free-base CK and, overall, that *cis*-Z-type CKs accumulated to much lower levels than those of *trans*-Z and iP-type CKs. However, it is striking that, compared to WT, the levels of *cis*-[9RMP]Z and *cis*-Z were higher while the corresponding *trans*-isomers were the same in soil-grown line 6.9, one of the transgenics which displayed the best stress performance. In fact, the *cis*-Z: *trans*-Z ratio was higher in all of the soil-grown transgenics.

Moreover, the rapid interconversion of base, ribosides and ribotides is a typical feature of CK metabolism, and plants are able to modulate the levels of *cis*-Z and *trans*-Z due to isomerization by *cis-trans* isomerase (Mok and Mok, 2001) so the observed increase in the concentration of *trans*-Z in *ABR17* transgenic lines (6.9 and 25.20) is possible by the interconversion of the *cis*-Z form generated by the tRNA degradation.

CKs are plant hormones that regulate many developmental and physiological processes in plants, including promotion of cell division, seed germination, chloroplast development, leaf senescence, lateral shoot development, vascular differentiation, nutrient mobilization and regulation of gene expression (Mok and Mok, 2001; Haberer and Kieber, 2002). Treatment with CKs has been shown to delay leaf senescence in many plants (van Staden *et al*., 1988) and decreased contents of CKs under drought, salinity and other stress conditions have been reported (Pospisilova *et al*., 2000). Furthermore, in tobacco isopentenyl transferase (*IPT)* transgenic plants with elevated CK content, an increase in proline and osmotin content was reported, suggesting that cytokinin may elicit responses similar to those induced by environmental stresses, including perhaps mediating tolerance to these stresses (Thomas *et al.*, 1995). Similarly, the maintenance of high concentrations of CKs in maize kernels during heat stress was suggested to be important in increasing thermo tolerance and providing yield stability (Cheikh *et al.*, 1994), and a role for CKs as antioxidants has also been suggested (Gidrol *et al.*, 1994; Brathe *et al.*, 2002).

We have previously demonstrated that the constitutive expression of pea *PR 10.1* cDNA, which is an RNase, in *B. napus* enhances endogenous CK concentrations (Srivastava *et al.*, 2006^{a}). In this study, we demonstrated that the constitutive expression of pea *ABR17* cDNA also results in an increase in endogenous concentrations of CK, which could be mediated via the observed RNase activity of this protein. However, the possibility of biosynthesis of these CKs (*cis*-Z, *trans*-Z and iP) by the tRNA independent pathway couldnot be ruled out. The possibility of tRNA degradation by the RNase activity of pea *ABR17* and enhanced biosynthesis of free CKs via this pathway remains speculative at this stage. Confirmation of this hypothesis will have to come from additional experiments, including the site-directed mutagenesis of those amino acids that are thought to be important for RNase activity, and the introduction of the mutant gene into *A. thaliana* followed by endogenous CK analysis. Additionally, the presence of iP and *cis*-Z in *A. thaliana*, as well as pea tRNA molecules must be demonstrated together with ABR17-mediated release of these CK moieties from the tRNA. Such experiments are currently in progress in our laboratory and will form the basis of future communications.

6.5. References

Antinov, J.F., Ritter, C.E., Pierpoint, W.S. and van Loon, N.C. (1980) Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. J Gen Virol **47:** 79–87.

Bantignies, B., Seguin, J., Muzac, I., Dedaldechamp, F., Gulick, P. and Ibrahim, R. (2000) Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. Plant Mol Biol **42:** 871–881.

Barnes, M.F., Tien, Ch.L. and Gray, J.S. (1980) Biosynthesis of cytokinins by potato cell cultures. Phytochemistry **19:** 409–412.

Barratt, D.H.P. and Clark, JA. 1993. A stress-induced, developmentally regulated highly polymorphic protein family in *Pisum sativum* L. Planta 191:7–17.

Bonhomme, F., Kurz, B., Melzer, S., Bernier, G. and Jacqmard, A. (2000) Cytokinin and gibberellin activate SaMADS A, a gene apparently involved in regulation of the floral transition in *Sinapis alba*. Plant J **24:** 103–111.

Brathe, A., Andresen, G., Gundersen, L.L., Malterud, K.E. and Rise, F. (2002) Antioxidant activity of synthetic cytokinin analogues: 6-alkynyl- and 6-alkenylpurines as novel 15-Lipoxygenase inhibitors. Bioorg Med Chem **10:** 1581–1586.

Bufe, A., Spangfort, M.D., Kahlert, H., Schlaak, M. and Becker, W.M. (1996) The major birch pollen allergen, Bet v 1, shows ribonuclease activity. Planta **199:** 413–415.

Cheikh, N. and Jones, R.J. (1994) Disruption of maize kernel growth and development by heat stress, role of cytokinin/abscisic acid balance. Plant Physiol **106:** 45–51.

Colditz, F., Nyamsuren, O., Niehaus, K., Eubel, H., Braun, H.P. and Krajinski, F. (2004) Proteomic approach: identification of *Medicago truncatula* proteins induced in roots after infection with the pathogenic oomycete *Aphanomyces euteiches*. Plant Mol Biol **55:** 109–120.

Crowell, D.N., John, M.E., Russell, D. and Amasino, R.M. (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol Biol **18:** 459–466.

Ferguson, B.J., Wiebe, E.M., Emery, R.J.N. and Guinel, F.C. (2005) Cytokinin accumulation and an altered ethylene response mediate the pleiotropic phenotype of the pea nodulation mutant R50 (sym16). Can J Bot **83:** 989–1000.

Fujimoto, Y., Nagata, R., Fukasawa, H., Yano, K., Azuma, M., Iida, A., Sugimoto, S., Shudo, K. and Hashimoto, Y. (1998) Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (*Vigna radiata*). Eur J Biochem **258:** 794–802.

Gidrol, X., Lin, W., Degousee, N., Yip, S. and Kush, A. (1994) Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. Eur J Biochem **224:** 21–28.

Haberer, G. and Kieber, JJ. (2002) Cytokinins. New insights into a classic phytohormone. Plant Physiol **128:** 354–362.

Hwang, I. and Sheen, J. (2001) Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature **413:** 383–389.

Iturriaga, E.A., Leech, M.J., Barratt, D.H.P. and Wang, T.L. (1994) Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. Plant Mol Biol **24:** 235–240.

Kakimoto, T. (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. Plant Cell Physiol **42:** 677-685.

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., Yamaguchi, S. and Sakakibara, H. (2004) Distinct isoprenoid origins of *cis*- and trans-zeatin biosyntheses in *Arabidopsis*. J Biol Chem **279:** 14049-14054.

Kav, N.N.V., Srivastava, S., Goonewardene, L. and Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity. Ann Appl Biol **145:** 217–230.

Klambt, D. (1992). The biogenesis of cytokinins in higher plants: our present knowledge. In: Kaminek M, Mok DWS, Zazimalova E, editors. Physiology and biochemistry of cytokinins in plants. The Hague The Netherlands: SPB–Academic Publ. p. 25–27.

Letham, D.S. and Palni, L.M.S. (1983) The Biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol **34:** 163–197.

Liu, X., Huang, B. and Banowetzb, G. (2002) Cytokinin effects on creeping bentgrass responses to heat stress I. shoot and root growth. Crop Sci **42:** 457–465.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2\text{-}\Delta^{\Delta Ct}$ method. Methods **25:** 402–408.

Mader, J.C., Turnbull, C.G.N. and Emery, R.J.N. (2003) Transport and metabolism of xylem cytokinins during lateral bud release in decapitated chickpea (*Cicer arietinum* L.) seedlings. Physiol Plant **117:** 118-129.

Markovic-Housely, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. and Breitender, H. (2003) Crystal structure of a hypoallergenic isoforms of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J Mol Biol **325:** 123–133.

Miyawaki, K., Matsumoto-Kitano, M. and Kakimoto, T. (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J **37:** 128–138.

Mogensen, J.E., Wimmer, R., Larsen, J.N. and Spangfort, M.D. (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J Biol Chem **277:** 684–692.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. and Yakovlev, G.I. (1994) High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193:** 470–472.

Mok, D.W. and Mok, M.C. (2001) Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol **52:** 89–118.

Park, C.J., Kim, K.J., Shin, R., Park, J.M., Shin, Y.C. and Paek, K.H. (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J **37:** 186–198.

Pasternak, O., Biesiadka, J., Dolot, R., Handschuh, L., Bujacz, G., Sikorski, M.M. and Jaskolski, M. (2005) Structure of a yellow lupin pathogenesis-related PR 10 protein belonging to a novel subclass. Acta Crystallogr D Biol Crystallogr **61:** 99–107.

Pospisilova, J., Synkova, H. and Rulcova, J. (2000) Cytokinins and water stress. Biol Plant **43:** 321–328.

Prinsen, E., Kaminek, M. and van Onckelen, H.A. (1997) Cytokinin biosynthesis: a black box? Plant Growth Reg **23:** 3–15.

Riefler, M., Novak, O., Strnad, M. and Schmulling, T. (2006) *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18:** 40–54.

Sakakibara, H. (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol **57:** 431–449.

Sikorski, M.M., Biesiadka, J., Kasperska, A.E., Kopcinska, J., Lotocka, B., Golinowski, W. and Legocki, A.B. (1999) Expression of genes encoding

PR 10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. Plant Sci **149:** 125–137.

Srivastava, S., Fristensky, B. and Kav, N.N.V. (2004) Constitutive expression of a PR 10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol **45:** 1320–1324.

Srivastava, S., Emery, R.J.N., Kurepin, L.V., Reid, D.M., Fristensky, B. and Kav, N.N.V. (2006[a]) Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels. Plant Growth Regul **49:** 17–25.

Srivastava, S., Rahman, M.H., Shah, S. and Kav, N.N.V. (2006[b]) Constitutive expression of the pea ABA-responsive (*ABR17*) cDNA confers multiple stress tolerance in *Arabidopsis thaliana.* Plant Biotech J **4:** 529–549.

Swaminathan, S., Bock, R. and Skoog, F. (1977) Subcellular localization of cytokinins in transfer ribonucleic acid. Plant Physiol **59:** 558–563.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H. and Mori, H. (2006) Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. Plant J **45:** 1028-1036.

Tewari, S., Brown, S.M., Kenyon, P., Balcerzak, M. and Fristensky, B. (2003) Plant defense multigene families. II Evolution of coding sequence and differential expression of PR 10 genes in *Pisum.* http://arXiv.org/q-bio.PE/0310038

Thomas, J.C., Smigocki, A.C. and Bohnert, H.J. (1995) Light-induced expression of ipt from *Agrobacterium tumefaciens* results in cytokinin accumulation and osmotic stress symptoms in transgenic tobacco. Plant Mol Biol **27:** 225–235.

van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Report **12:** 245–264.

van Loon, L.C. and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol **55:** 85–97.

Van Staden, J., Cook, E.L. and Nooden, L.D. (1988) Cytokinins and senescence. In: Nooden LD, Leopold AC, editors. Senescence and aging in plants. San Diego: Academic–Press. p 281–328.

Warner, S.A.J., Gill, A. and Draper, J. (1994) The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. Plant J **6:** 31–43.

Wu, F., Li, Y.K., Chang, S., Zhou, Z., Wang, F., Song, X., Lin, Y. and Gong, W. (2002) Purification, characterization and preliminary crystallographic studies of a PR 10 protein from *Pachyrrhizus erosus* seeds. Acta Crystal D **58:** 2165–2167.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T. and Mizuno, T. (2001) The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol **42:** 1017–1023.

7. General Discussion

Environmental stresses such as salinity, drought and temperature extremes severely limit crop productivity worldwide (Boyer, 1982) and it is estimated that by the middle of the 21st century, widespread salinity may cause serious salinization of more than 50% of all arable lands (Wang *et al.*, 2003). Plant responses to salt stress are very complex and approaches to generate salinity-tolerant plants via conventional breeding have had limited success (Zhu, 2002), whereas enhancing plant tolerance to abiotic stresses by introducing genes have demonstrated significant success (Apse *et al.*, 1999). Identification of novel genes or proteins that can be utilized to confer abiotic stress tolerance is a challenging task, but information from plant genome projects and the advent of techniques such as microarrays (Ozturk *et al.*, 2002; Wong *et al.*, 2006) and proteomics (Salekdeh *et al.*, 2002; Agrawal *et al.*, 2005) has resulted in the identification of many targets that can be utilized to engineer plant abiotic stress tolerance. In many legume species, proteins belonging to the PR 10 family are present in higher concentration in roots (Sikorski *et al.*, 1999; Bantignies *et al.*, 2000) and the levels of these proteins are elevated by many abiotic stresses (Liu and Ekramoddoullah, 2003; Hashimoto *et al.*, 2004; Kav *et al.*, 2004), suggesting a possible role for them in ameliorating the deleterious effects of stress. Based on these observations we hypothesized that in addition to other protein changes, salt stress may also increase the levels of PR 10 proteins in pea (*Pisum sativum*) roots.

Microarray and proteomics are useful techniques for transcript/protein expression analysis. However, due to protein turnover and post-translational modifications, only the study of proteins provides good information on their abundance and activity (Gygi *et al.,* 1999; Zivy and Vienne, 2000). Therefore, we used a comprehensive proteomics-based approach to test our hypothesis. Here, proteome analysis using two-dimensional electrophoresis and mass spectrometry revealed an increase in the levels of many members of PR 10 proteins, as well as other proteins such as superoxide dismutase (SOD) and nucleoside diphosphate kinase (NDPK) in salt-stressed pea roots (Kav *et al.*, 2004). Comparison of the proteome of a salinity-tolerant *Arachis hypogaea* callus with a salinity-sensitive line revealed several unique or significantly elevated PR 10 proteins in salinity-tolerant lines (Appendix I, Srivastava *et al.*, 2006[a]). The results from both salinity-stressed pea roots and salinity-tolerant peanut callus suggested a crucial role of PR 10 proteins in ameliorating the deleterious effects of salinity.

The pea PR 10 family consists of five members such as *pi49* (*10.1*), *pi176* (*10.2*), *drrc* (*10.3*), *ABR17* (*10.4*) and *ABR18* (*10.5*) (Tewari *et al.*, 2003) and at least four members of the group (10.1, 10.2, 10.4, 10.5) were up-regulated in salt-stressed pea roots (Kav *et al.*, 2004). The sequence alignment of PR 10.1 with PR 10.2 and PR 10.1 with PR 10.4 proteins revealed an overall identity of 96% and 56%, respectively. Based on the sequence alignments, as well as the abundance of PR 10 proteins (Kav *et al.,* 2004) we investigated the role of pea PR 10.1 and ABR17 (PR 10.4) proteins. We hypothesized that they play a crucial role in mediating abiotic stress tolerance. Testing mutants of *Arabidopsis thaliana* which lacked these proteins would have been a logical first choice to test this hypothesis; however, database searches did not reveal presence of *PR 10* genes in *A. thaliana,* although their distant homologues, the major latex proteins (MLPs) were present; therefore, we evaluated plants constitutively expressing *PR 10* genes in order to test our hypotheses.

Canola (*Brassica napus* L.) is an economically important crop grown on the Canadian prairies on 25-30% of the annual crop acreage in Western Canada. Wang *et al.* (1999) constitutively expressed the pea *PR 10.1* gene in *B. napus* and tested resistance against blackleg disease (*L. maculans*). The transgenic *B. napus* lines did not exhibit resistance to blackleg disease but the availability of this transgenic material enabled us to quickly test our hypothesis. As with *A. thaliana,* the database searches did not reveal any reported *PR 10* genes among the *B. napus* sequences available. The presence of *PR 10.1* transcript and protein was confirmed in *B. napus* lines using RT-PCR and two-dimensional electrophoresis, followed by mass spectrometry. The hypothesis that PR 10 proteins play a crucial role in ameliorating the deleterious effects of salinity was tested by germinating the *PR 10.1* transgenic *B. napus* seeds in the presence of salt which demonstrated that transgenic *B. napus* seedlings were able to germinate and survive better under these conditions when compared to the wild type (Srivastava *et al.*, 2004).

In order to evaluate the potential of another gene of the *PR 10* family, *ABR17* (or *PR 10.4*), in a different species and to understand the role of PR 10 in detail, the model plant *A. thaliana* was transformed. This was due to the availability of wealth of information for this model plant and its ability to be quickly transformed. As indicated earlier, there is only ~56% sequence homology between PR 10.1 and ABR17 at the amino acid level and it was interesting to see whether these two proteins elicited the same effects *in planta*. Transcript and protein expression were confirmed in transgenic *A. thaliana* lines and seeds were tested for abiotic stress tolerance. The transgenic *A. thaliana* seeds constitutively expressing *ABR17* cDNA had enhanced tolerance to salinity and cold stress (Srivastava *et al.*, 2006[b]). These results demonstrated the utility of at least these two *PR 10* genes and perhaps all *PR 10* genes in enhancing germination and conferring abiotic stress tolerance (Srivastava *et al.*, 2004; Srivastava *et al.*, 2006[b]).

In order to test our hypothesis that pea PR 10 proteins possess ribonuclease activity, the cDNAs encoding *PR 10.1* or *ABR17* were expressed in *Escherichia coli* and the recombinant proteins were purified to homogeneity using Ni-NTA and DEAE-Sepharose chromatography. An in-solution as well as an in-gel activity assay, clearly demonstrated that recombinant pea PR 10.1 and ABR17 proteins possess RNase activity (Srivastava *et al.*, 2006[c]; Srivastava *et al.*, 2006[d]). In addition, analysis of the RNase activity of salinity-sensitive and salinity-tolerant *A. hypogaea* lines revealed that the salinity-tolerant *A. hypogaea* line possesses more RNase activity in the region corresponding to molecular weight of PR 10 protein (Appendix II). Furthermore, the availability of another purified recombinant protein Pm PR 10 (*Pinus monticola* cold-induced PR 10 protein) led us to test the RNase activity of this protein. Results from the RNase in-gel assay demonstrated that Pm PR 10 protein does not possess RNase activity. However, in this case the protein had been stored for an extended period of time and may have lost its activity (Appendix II). PR 10 proteins from a few plant species including legumes have been reported to possess RNase activity (Srivastava *et al.*, 2006[c]); however, the biological importance of this activity *in planta* and, its relevance to mediating abiotic stress responses is still unclear. RNase activity analysis of *P. sativum*, *A. hypogaea* and *P. monticola* PR 10 proteins in this study suggested that RNase activity may not be a general feature of PR 10 proteins and that the significance of RNase activty in the evolutionary divergence of different plant species requires further investigation. However, it is clear that the two pea PR 10 proteins tested in this study possess RNase activity, and based on sequence similarities with the other three members of the pea PR 10 protein family it is possible to conclude that all pea PR 10 proteins possess RNase activity.

RNase activity plays an important role in plant development by modifying the RNA levels, and thereby influencing protein synthesis (Tvorus, 1976). Levels of RNase have been demonstrated to be altered in response to endogenous or exogenous stimuli (Dodds *et al.*, 1996; Liu *et al.*, 2006; Srivastava *et al.*, 2006^c), including salinity and water stress (Rouxel *et al.*, 1989). The RNase activity of PR 10 proteins may play an important role in defence against pathogen infections and such a role was demonstrated by studies on the antifungal and antibacterial activity of SsPR 10 from *Solanum surattense* (Liu *et al.*, 2006), and antifungal and antiviral activity of CaPR 10 from pepper (Park *et al.*, 2004).

Salt stress inhibits seed germination and seedling growth mainly by delaying mobilization of seed reserves (Gomes Filho *et al.*, 1983), and RNase activity plays a crucial role in RNA turn over and mobilization from storage tissues that are important for seed germination and seedling establishment (Gomes Filho *et al.*, 1983; Gomes Filho and Sodek, 1988). Therefore, during germination under normal or saline conditions induction of PR 10 proteins and consequent RNase activity may play a crucial role by preventing the delayed seed reserve mobilization. The RNase activity of SPE16 (another PR 10 protein), which is a constitutive protein rather than a protein induced by pathogen invasion or other elicitors, suggests that RNase activity of PR 10 proteins may also play a crucial role in the regulation of plant growth and development even under normal conditions (Wu *et al.*, 2003).

The PR 10 family contains a highly conserved glycine rich loop which resembles the phosphate-binding loop motif (GXGGXGXXK) present in many nucleotide-binding proteins. This P-loop is suggested to play an important role in the biological function of PR 10 by binding the phosphate group of RNA in order to mediate the RNase activity (Saraste *et al.*, 1990; Wu *et al.*, 2003). Structural studies in the PR 10 homologue Bet v 1 predicted that three conserved amino acids Glu-96, Glu-148, and Tyr-150 are involved in RNase activity (Moiseyev *et al.*, 1997), site-directed mutagenesis of these conserved amino acids in SPE 16, as well as those in the P-loop, affected RNase activity, thereby providing additional evidence for the importance of these amino acids for the RNase activity of these PR 10 proteins (Wu *et al.*, 2003). In addition, similarities of PR 10 proteins with cytokinin-specific binding proteins at the primary structural level have led many authors to speculate that PR 10 proteins may be binding ligands such as cytokinins (CKs; Fujimoto *et al.*, 1998; Mogensen *et al.*, 2002). In addition, *in vitro* studies have demonstrated that some PR 10 proteins indeed interact with CKs as well as several other ligands (Biesiadka *et al.*, 2002; Markovic-Housley *et al.*, 2003).

PR 10 proteins have several putative phosphorylation sites, and using Pro-Q-diamond phosphoprotein specific stain we demonstrated that Ara h 8 proteins in peanut (Srivastava *et al.*, 2006[a]) are phosphorylated. Park *et al.* (2004) reported that the Capsicum PR 10 protein is phosphorylated upon TMV inoculation and the phosphorylated protein functions as an RNase that is able to cleave viral RNA. These observations suggest that phosphorylation of PR 10 proteins is important for their biological activities, and may give specificity to certain RNA substrates or against non-self RNA; otherwise, their explicit activity would be detrimental for plants (McGee *et al.*, 2001; Park *et al.*, 2004). Specificity of RNase activity of PR 10 is evident in proteins such as Bet v 1 from major birch pollen allergens where RNase activity in pollen grains could be related to the defense reaction of pollen against pathogen attack (Breiteneder *et al.*, 1989) or in recognition and self-incompatibility during fertilization (Huang *et al.*, 1994; Swoboda *et al.*, 1995) or, as the results from the studies described in this dissertation have demonstrated (at least for seeds) they may play a role in seed germination. Specificity of PR 10 was also evident in a previous transgenic study where overexpression of *PR 10* gene in potato (Constabel *et al.*, 1993) or canola (Wang *et al.*, 1999^a) does not exhibit any phenotypic abnormality.

The RNase activity of PR 10 proteins, the presence of CK moieties in some tRNAs (Prinsen *et al.*, 1997; Hwanga and Sakakibarab, 2006), the interaction of some PR 10 proteins with CKs, and phosphorylation-mediated specificity of the RNase activity all led us to speculate that the RNase activity and CK binding are related. For instance, the CK-binding activity of some PR 10 proteins may be that the RNase activity is specific for the CK-moieties present in tRNAs, and this specific binding is mediated via phosphorylation. In order to test whether the overexpression of *PR 10* genes modulates endogenous CK levels we determined the concentrations of CKs, as well as other phytohormones, in transgenic *B. napus* expressing the *PR 10.1* cDNA and in transgenic *A. thaliana* expressing the *ABR17* cDNA. Results from those studies revealed an increase in CK, a decrease in abscisic acid (ABA) and no significant changes in gibberellin (GA) or indoleacetic acid (IAA) levels in 7-day-old *B. napus* seedlings (Srivastava *et al.*, 2006[c]). In the case of *ABR17 A. thaliana* seedlings, we observed an increase in total CK levels, and specifically those specific forms of CKs that would be expected as a result of tRNA hydrolysis (Srivastava *et al.*, 2006[d]). There was a slight decrease in IAA and GA4. We were unable to detect ABA and GA1 (Appendix III).

CKs are plant hormones that regulate many developmental and physiological processes in plants, and they antagonize many physiological processes induced by water stress (Mok and Mok, 2001; Haberer and Kieber, 2002). An increase in CK levels in *B. napus* and *A. thaliana* transgenic lines suggested that pea PR 10 activity modulates the CK levels (Srivastava *et al.*, 2006[c]; Srivastava *et al.*, 2006[d]). Gene expression analysis of a cytokinin-response regulator *ARR5* revealed a 4-9 fold increase in three *ABR17 A. thaliana* transgenic lines, and by exogenous application of CKs germination of *A. thaliana* seeds under abiotic stress conditions increased by exogenous application of CK up to an extent comparable to that of *ABR17 A. thaliana* transgenic plants (Srivastava *et al.*, 2006[d]).

The RNase activity associated with pea PR 10 proteins and the modulation of CK levels suggests that RNase activity of PR 10 proteins is responsible for the increase in endogenous CK levels (Srivastava *et al.*, 2006[c]; Srivastava *et al.*, 2006[d]). Support for the hypothesis is derived from the suggestion that tRNAs are possible source of endogenous CKs (Swaminathan *et al.*, 1977); it has been estimated that tRNAs contribute 40–50% of CK pool in plants (Barnes *et al.*, 1980; Letham and Palni, 1983; Prinsen *et al.*, 1997). The CK profile of *PR 10.1 B. napus* and *ABR17 A. thaliana* transgenic plants, *ARR5* expression analysis and phenocopying experiments by exogenous application provided some evidence for such a hypothesis (Srivastava *et al.*, 2006^{c}; Srivastava *et al.*, 2006^{d}). However a direct experimental proof is required to confirm such a hypothesis, and future research in this direction may enhance our understanding of PR 10 biology as well as that of CK biosynthesis pathways.

7.1. Future prospects

Results presented in this study have established a very crucial role of *PR 10* genes in engineering abiotic stress tolerance in plants and suggested that RNase activity of PR 10 proteins may modulate the CK levels. Knowledge gained from the studies described in this dissertation enhanced our understanding of some of the roles of PR 10 proteins *in planta*. However, many questions still remain and may be answered in the future by investigating other aspects of PR 10 biology including:

1. Testing the utility of five members of pea *PR 10* genes, individually and in combination, in other crops (e.g. wheat, rice and elite varieties of canola) for their ability to enhance germination under abiotic stress conditions.

2. Additional experiments to provide direct evidence for the RNase and CK hypothesis:

(a) Producing mutant genes via site-directed mutagenesis of amino acids such as Glu-96, Glu-148, Tyr-150 and P-loop, which have been predicted to be important for RNase activity (Moiseyev *et al.*, 1997; Wu *et al.*, 2003), followed by introduction of mutant gene (s) individually and in combination into *A. thaliana* and *B. napus* in order to test their influence on germination and stress tolerance, as well as analyzing their endogenous CK profile.

(b) Isolating tRNA from *A. thaliana*, *B. napus* and *P. sativum*, their (i) hydrolysis; (ii) incubation with PR 10 proteins (iii) feeding with radiolabels, and analysis of endogenous CK profile to investigate the presence of CK moieties such as iP and *cis*-Z which have been predicted to be source of tRNA degradation (Prinsen *et al.*, 1997; Sakakibara, 2006);-and,

(c) Testing the possibility of CK biosynthesis by tRNA independent pathways such as isopentenyl transferase (*IPT*) and reduced degradation/oxidation by cytokinin oxidase (*CKX*).

3. RNase activity of PR 10 proteins and its ability to modulate CK levels seem very important but diverse role (s) played by this group of proteins are evident by their role in sporopollenin pathway (PR 10 protein in the tapetum of lily anthers; Balsamo *et al.*, 1995; Wang *et al.*, 1999^{b}), role in phenylpropanoid biosynthesis (PR 10 protein in Asparagus AoPR1; Warner *et al.*, 1994), a role in signal transduction (Kav *et al.*, 2004), a role similar to dehydrin/LEA like proteins (Pneuli *et al.*, 2002), and ability to bind various ligand (e.g. cytokinins, fatty acids, flavonoids and brassinosteroids;

Fujimoto *et al.*, 1998; Mogensen *et al.*, 2002; Markovic-Housely *et al.*, 2003; Koistinen *et al.*, 2005), which suggests that PR 10 proteins may be multifunctional.

4. From the literature it is evident that CK affects the levels of Nitrate Reductase (NR) (Ferrario-Mery *et al.*, 1998; Yu *et al.*, 1998). A preliminary analysis by semi-quantitative PCR using primers specific for *NR1* and *NR2* revealed increased transcript levels in *ABR17* transgenic *Arabidopsis* lines. Further analysis of *NR* expression in PR 10 transgenic plants, their nitrogen utilization efficiency and a possible relation between PR 10, CK and NR levels requires further detailed investigation.

5. Testing the involvement of PR 10 proteins in signal transduction pathways.

6. Analyzing RNase activity of different PR 10 proteins within the context of evolutionary divergence of different plant species.

7.2. Conclusions

Discovery and use of novel targets to confer abiotic stress tolerance in plants has been the subject of ongoing efforts and microarray and proteomics-based techniques have accelerated the pace of such research. In this research, we used a proteomics-based approach to investigate the proteome-level changes in salt-stressed pea roots and salinity-tolerant *A. hypogaea* callus. Results from these studies demonstrated that PR 10 proteins are expressed at higher levels under saline conditions. Based on these results, a crucial role of *PR 10* gene (s) in mediating responses to salinity and other abiotic stresses was hypothesized and transgenic lines overexpressing pea *PR 10.1* gene in *B. napus* and *ABR17* gene in *A. thaliana* were tested for their ability to germinate and grow under various abiotic stress conditions. Results from these investigations demonstrated enhanced germination and tolerance to multiple abiotic stresses in PR 10 transgenic plants.

The biological activity of PR 10 protein was tested by overexpressing the cDNA of *PR 10.1* and *ABR17* in *E. coli* and purifying the recombinant proteins to homogeneity. The purified proteins were used to test their RNase activity which demonstrated that pea PR 10 proteins possess RNase activity. Transgenic plants were characterized to investigate the effects of constitutive expression of *PR 10* gene (s). Analysis of their endogenous phytohormones levels revealed a significant increase in CK levels. The increased CK level was also accompanied by an increase in the transcript levels of *ARR5* which is a primary cytokinin response regulator. Under abiotic stress conditions, exogenous application of various CKs enhanced germination of wild type *A. thaliana* to a level similar to *ABR17* transgenics, suggesting a role of CK in mediating such responses.

Results presented in this study also suggest that PR 10 proteins have a very important role in plant growth and development and it may open a novel avenue to engineer abiotic stress tolerance in plants. Although the precise mechanism by which PR 10 is able to modulate CK levels remains a conjecture at this point, it is possible that the RNase activity of these proteins contributes to the endogenous pools of CK. The significance of RNase activity in relation to CK and the multifunctional role (s) of PR 10 proteins needs further investigation and may further enhance our understanding of the biology of PR 10 proteins *in planta*.

7.3. References

Agrawal, G.K., Yonekura, M., Iwahashi, Y., Iwahashi, H. and Rakwal, R. (2005) System, trends and perspectives of proteomics in dicot plants. Part III: Unraveling the proteomes influenced by the environment, and at the levels of function and genetic relationships. J Chromatogr B Analyt Technol Biomed Life Sci **815:** 137–145.

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science **285:** 1256–1258.

Balsamo, R.A., Wang, J.-L., Eckard, K.J., Wang, C.-S. and Lord, E.M. (1995) Immunogold localization of a developmentally regulated, tapetal-specific, 15 kDa lily anther protein. Protoplasma **189:** 17–25.

Bantignies, B., Seguin, J., Muzac, I., Dedaldechamp, F., Gulick, P. and Ibrahim, R. (2000) Direct evidence for ribonucleolytic activity of a PR 10 like protein from white lupin roots. Plant Mol Biol **42:** 871–881.

Barnes, M.F., Tien, Ch.L. and Gray, J.S. (1980) Biosynthesis of cytokinins by potato cell cultures. Phytochemistry **19:** 409–412.

Biesiadka, J., Bujacz, G., Sikorski, M.M. and Jaskolski, M. (2002) Crystal structures of two homologous pathogenesis-related proteins from yellow lupine. J Mol Biol **319:** 1223–1234.

Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. and Breitenbach, M. (1989) The gene coding for the major birch pollen allergen Betv1, is highly homologous to a pea disease resistance response gene, EMBO J. **8:** 1935–1938.

Boyer, J.S. (1982) Plant productiviy and the environment. Science **218:** 443–448.

Constabel, C. P., Bertrand, C. and Brisson, N. (1993) Transgenic potato plants overexpressing the pathogenesis-related STH-2 gene show unaltered susceptibility to Phytophthora infestans and potato virus X. Plant Mol. Biol. **22:** 775-782.

Dodds, P.N., Clarke, A.E. and Newbigin, E. (1996) Molecular characterisation of an S-like RNase of Nicotiana alata that is induced by phosphate starvation. Plant Mol Biol **31:** 227–238.

Ferrario-Mery, S., Valadier, M.H. and Foyer, C.H. (1998) Overexpression of nitrate reductase in tobacco delays drought-induced decreases in nitrate reductase activity and mRNA. Plant Physiol **117:** 293–302.

Fujimoto, Y., Nagata, R., Fukasawa, H., Yano, K., Azuma, M., Iida, A., Sugimoto, S., Shudo, K. and Hashimoto, Y. (1998) Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (*Vigna radiata*). Eur J Biochem **258:** 794–802.

Gomes Filho, E., Prisco, J.T., Campos, F.A.P. and Eneas Filho. (1983) Effects of NaCl salinity in vivo and in vitro on ribonuclease activity of *Vigna unguiculata* cotyledons during germination. Physiol Plant **59:** 183–188.

Gygi, S.P., Rochon, Y., Franza, B.R. and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol **19:** 1720–1730.

Gomes Filho, E. and Sodek, L. (1988) Effect of salinity on ribonuclease activity of *Vigna unguiculata* cotyledons during germination. J Plant Physiol **132:** 307–311.

Haberer, G. and Kieber, J.J. (2002) Cytokinins. New insights into a classic phytohormone. Plant Physiol **128:** 354–362.

Hashimoto, M., Kisseleva, L., Sawa, S., Furukawa, T., Komatsu, S. and Koshiba, T. (2004) A novel rice PR 10 protein, RSOsPR 10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. Plant Cell Physiol **45:** 550–559.

Huang, S., Lee, H.-S., Karunanandaa, B. and Kao, T.-h. (1994) Ribonuclease activity of Petunia inflata S proteins is essential for rejection of self-pollen. Plant Cell **6:** 1021–1028.

Hwanga, I. and Sakakibarab, H. (2006) Cytokinin biosynthesis and perception. Physiol Plant **126:** 528–538.

Kav, N.N.V., Srivastava, S., Goonewardene, L. and Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity. Ann Appl Biol **145:** 217–230.

Koistinen, K.M., Soininen, P., Venalainen, T.A., Hayrinen, J., Laatikainen, R., Perakyla, M., Tervahauta, A.I. and Karenlampi, S.O. (2005) Birch PR-10c interacts with several biologically important ligands. Phytochemistry **66:** 2524–2533.

Letham, D.S. and Palni, L.M.S. (1983) The Biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol **34:**163–197.

Liu, J.J. and Ekramoddoullah, A.K. (2003) Root-specific expression of a western white pine PR 10 gene is mediated by different promoter regions in transgenic tobacco Plant Mol Biol **52:** 103–120.

Liu, X., Huang, B., Lin, J., Fei, J., Chen, Z., Pang, Y., Sun, X. and Tang, K. (2006) A novel pathogenesis-related protein (SsPR 10) from *Solanum surattense* with ribonucleolytic and antimicrobial activity is stress- and pathogen-inducible. J Plant Physiol **163:** 546–556.

Markovic-Housely, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. and Breitender, H. (2003) Crystal structure of a hypoallergenic isoforms of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J Mol Biol **325:** 123–133.

McGee, J.D., Hamer, J.E. and Hodges, T.K. (2001) Characterization of a PR-10 pathogenesis-related gene family iInduced in rice during infection with *Magnaporthe grisea*. MPMI **14:** 877–886.

Mogensen, J.E., Wimmer, R., Larsen, J.N. and Spangfort, M.D. (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J Biol Chem **277:** 684–692.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. and Yakovlev, G.I. (1994) High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193:** 470–472.

Mok, D.W. and Mok, M.C. (2001) Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol **52:** 89–118.

Ozturk, Z.N., Talame, V., Deyholos, M., Michalowski, C.B., Galbraith, D.W., Gozukirmizi, N., Tuberosa, R. and Bohnert, H.J. (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol **48:** 551–573.

Park, C.J., Kim, K.J., Shin, R., Park, J.M., Shin, Y.C. and Paek, K.H. (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J **37:** 186–198.

Pnueli, L., Hallak-Herr, E., Rozenberg, M., Cohen, M., Goloubinoff, P., Kaplan, A. and Mittler, R. (2002) Mechanisms of dormancy and drought tolerance in the desert legume *Retama raetam*. Plant J **31:** 319–330.

Prinsen, E., Kaminek, M. and van Onckelen, H.A. (1997) Cytokinin biosynthesis: a black box? Plant Growth Reg **23:** 3–15.

Rouxel, M.F., Billard, J. P. and Boucaud, J. 1987. Effect of NaCl salinity in vivo and in vitro on ribonuclease activity in the halophyte *Suaeda maritime*. Physiol Plant **69:** 330–336.

Sakakibara, H. (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol **57:** 431–449.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. and Bennett, J. (2002) Proteomic analysis of rice leaves during drought stress and recovery. Proteomics **2:** 1131–1145.

Saraste, M., Sibbald, P.R. and Wittinghofer., A. (1990) The P-loop – a common motif in ATP- and GTP-binding proteins, Trends Biochem. Sci. **15:** 430–434.

Sikorski, M.M., Biesiadka, J., Kasperska, A.E., Kopcinska, J., Lotocka, B., Golinowski, W. and Legocki, A.B. (1999) Expression of genes encoding PR 10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. Plant Sci **149:** 125–137.

Srivastava, S., Fristensky, B. and Kav, N.N.V. (2004) Constitutive expression of a PR 10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol **45:** 1320–1324.

Srivastava, S., Jain, S., Sarin, N. and Kav, N.N.V. (2006[a]) Increase in levels of pathogenesis-related (PR) 10 proteins in saline-tolerant *Arachis hypogaea* L. calli. Plant Physiol Bioche **44:** 253–259.

Srivastava, S., Rahman, M.H., Shah, S. and Kav, N.N.V. (2006[b]) Constitutive expression of the pea ABA-responsive (ABR17) cDNA confers multiple stress tolerance in *Arabidopsis thaliana.* Plant Biotech J **4:** 529–549.

Srivastava, S., Emery, R.J.N., Kurepin, L.V., Reid, D.M., Fristensky, B. and Kav, N.N.V. (2006[c]) Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels. Plant Growth Regul **49:** 17–25.

Srivastava, S., Emery, R.J.N., Rahman, M.H. and Kav, N.N.V. (2006[d]) A crucial role for cytokinins in pea ABR17-mediated enhanced germination and early seedling growth of *Arabidopsis thaliana* under saline and low temperature stresses. J Plant Growth Regul (*in press*).

Swaminathan, S., Bock, R. and Skoog, F. (1977) Subcellular localization of cytokinins in transfer ribonucleic acid. Plant Physiol **59:** 558–563.

Swoboda, I., Hoffmann-Sommergruber, K., Riordain, G. O, Scheiner, O., Heberle-Bors, E. and Vicente, O. (1996) Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. Physiol Plant **96:** 433–438.

Tewari, S., Brown, S.M., Kenyon, P., Balcerzak, M. and Fristensky, B. (2003). Plant defense multigene families. II Evolution of coding sequence and differential expression of PR 10 genes in Pisum. http://arXiv.org/q-bio.PE/0310038

Tvorus, E.K. Plant ribonucleases. (1976) Soviet Plant Physiology **23:** 882–889.

Wang, Y., Nowak, G., Culley, D., Hadwiger, L.A. and Fristensky, B. (1999[a]) Constitutive expression of pea defense gene DRR 206 confers resistance to Blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). Mol Plant Microbe Interact **12:** 410–418.

Wang, C.S., Huang, J.-C. and Hu, J.-H. (1999[b]) Characterization of two subclasses of PR 10 transcripts in lily anthers and induction of their genes through separate signal transduction pathways. Plant Mol Biol **40:** 807–814.

Wang, W., Vinocur, B. and Altman, A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218:** 1–14.

Warner, S.A.J., Gill, A. and Draper, J. (1994) The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. Plant J **6:** 31–43.

Wong, C.E., Li, Y., Labbe, A., Guevara, D., Nuin, P., Whitty, B., Diaz, C., Golding, G.B., Gray, G.R., Weretilnyk, E.A., Griffith, M. and Moffatt, B.A. (2006) Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in Thellungiella, a close relative of *Arabidopsis*. Plant Physiol **140:** 1437–1450.

Wu, F., Yan, M., Li, Y., Chang, S., Song, X., Zhou, Z. and Gong, W. (2003) cDNA cloning, expression, and mutagenesis of a PR-10 protein SPE-16 from the seeds of *Pachyrrhizus erosus*. Biochem Biophys Res Commun **312:** 761–766.

Yu, X., Sukumaran, S. and Mrton, L. (1998) Differential expression of the *Arabidopsis* nia1 and nia2 genes. cytokinin-induced nitrate reductase activity is correlated with increased nia1 transcription and mrna levels Plant Physiol **116:** 1091–1096.

Zhu, J.-K. (2002) Salt and drought stress signal transduction in plants. Annual Review of Plant Biol **53:** 247–273.

Zivy, M. and de Vienne, D. (2000) Proteomics: a link between genomics, genetics and physiology. Plant Mol Biol **44:** 575–580.

Appendix I

Proteomics reveals elevated levels of PR 10 proteins in saline-tolerant peanut (*Arachis hypogaea*) calli

AI.1. Introduction

Environmental stresses such as drought and salinity severely limit agricultural productivity by reducing average crop yield by more than 50% (Boyer, 1982). Salinity is particularly a global concern due to the widespread salinization of soils, which is estimated to reach a staggering 50% of all arable land by the year 2050 (Wang *et al.*, 2003). Salinity-induced damage to plants include membrane disorganization, increase in levels of toxic metabolites, inhibited nutrient uptake and photosynthesis, generation of reactive oxygen species (ROS) and ultimately cell and plant death (Hasegawa *et al.*, 2000). Due to this intricate and complex nature of plant responses to salinity, the quest for salinity-tolerant plants that are generated via conventional breeding has remained largely unsuccessful (Zhu, 2002) whereas, biotechnological approaches have had some success (Apse *et al.*, 1999; Nagaoka *et al.*, 2003)

The relatively homogenous population of cells provided by *in vitro* cultures of plant cells offers an excellent system to investigate the effects of salinity stress.

A version of this chapter has been published: ***Srivastava S.****, Jain S., Sarin N. and Kav N.N.V. (2006) Increase in levels of pathogenesis-related (PR) 10 proteins in saline-tolerant Arachis hypogaea L. calli.* ***Plant Physiology and Biochemistry*** *44: 253-259.*

Furthermore, the growth of plant cells in culture under prolonged salinity stress often results in tolerance and therefore may offer clues as to the molecular mechanisms that may be crucial to the development of tolerance (Singh *et al.*, 1985; Murota *et al.*, 1994; Jain *et al.*, 2001). In today's post-genomic era, techniques that investigate changes in the protein component of the genome, i.e. the proteome, are being increasingly used in various disciplines in order to investigate molecular changes that occur in response to stresses (Salekdeh *et al.*, 2002; Kav *et al.*, 2004; Subramanian *et al.*, 2005). In this report we describe the characterization of proteome-level differences between a salinity-tolerant (ST) and –sensitive (SS) callus cell lines of *Arachis hypogaea* with a view of further understanding the molecular differences between the two lines that may be responsible for the higher tolerance to NaCl.

AI.2. Materials and methods

AI.2.1 Plant material

Salinity-tolerant *A. hypogaea* callus lines from leaf explants were developed as previously described through a stepwise selection process that involved challenging the callus initially with 50 mM NaCl in semi solid MS medium (Murashige and Skoog, 1962) supplemented with 1mg/l each of BAP and NAA (MSB1N1) and gelled with 0.8% agar followed by a gradual increase in NaCl concentration up to 200 mM (Jain *et al.*, 2001). The surviving, actively growing and green microcalli were isolated after two weeks and placed on salt free semi solid MSB1N1 medium for an additional two weeks to allow the cells to recover. After the recovery period, the cells were continuously maintained in MSB1N1 medium supplemented with 200 mM NaCl and sub-cultured every 15 days. In order to assess the inhibitory effect of NaCl on the growth of the tolerant and sensitive cell lines they were transferred to semi-solid MSB1N1 media containing 0-300 mM NaCl. The fresh weight of the cell lines on day 1 at 0 mM NaCl was considered to be 100% and the change in fresh weight on day 12 was calculated and expressed as a % of the initial value. All the experiments were performed with callus tissue harvested on 12th day of subculture in semisolid MSB1N1 medium and the data analyzed using Student's *t*-test.

AI.2.2. Protein Extraction, two-dimensional electrophoresis and Mass Spectrometry

Protein extracts for two-dimensional electrophoresis were prepared from three independent lyophilized callus tissue as described in (Srivastava *et al.*, 2004) with some modifications. Modification included a desalting step by using ReadyPrep 2D cleanup kit (Bio-Rad) according to the supplier's instructions. Two-dimensional electrophoresis, staining with Coomassie blue or Silver Stain Plus kit (Bio-Rad) and analysis of gel images using the PDQuest software (Bio-Rad) were performed as described in Srivastava *et al.* (2004). A thorough analysis of each region of each of the three SS and ST gels was performed and replicate groups were created using PDQuest software. The unique spots as well as those whose levels were significantly increased

($P<0.05$ using Student *t*-test) were selected for ESI-Q-ToF MS/MS analysis (Srivastava *et al.*, 2004).

AI.2.3. Phosphoproteome analysis

The phosophoproteome was analyzed using Pro-Q Diamond Phosphoprotein stain (Molecular probes, Eugene, OR). The protein extract prepared for two-dimensional gel analysis was delipidated and desalted as recommended by the manufacturer and the resulting pellet was dissolved in rehydration/sample buffer (Bio-Rad). This protein sample (300 μg) was used to perform two-dimensional electrophoresis as described above. Following electrophoresis the gels were immersed in 500 mL of fixing solution (50% methanol and 10% TCA) and incubated at room temperature with gentle agitation for 1 h. The fixing step was repeated once with fresh fixing solution to ensure complete removal of SDS from the gels. The gels were subsequently rinsed with water and incubated in 500 mL water for 15 min. This process was repeated four times to ensure that all the methanol and TCA was removed. Following these washing steps, the gels were incubated at room temperature in the dark in 500 ml of Pro-Q Diamond Phosphoprotein gel stain for 2 h with gentle agitation. Gels were destained by placing them in 500 mL of destaining solution and incubation in dark for 1 h with gentle agitation. The destaining process was repeated four times to get the optimal signal intensity and the images of the destained gels were captured using a UV transilluminator. This experiment was repeated at least three times.

AI.3. Results and discussion

A salinity-tolerant cell line of *Arachis hypogaea* cv. JL 24 has been obtained by exposing the callus cells to NaCl as previously described (Jain *et al.*, 2001) and the tolerant cell line (ST) appears to grow better on NaCl than the sensitive lines (SS). Appearance of the SS and ST lines cultured on semi-solid media are shown in Figure AI-1A and the effects of NaCl on fresh weight of both lines are shown in Figure AI-1B. It is evident that the ST line appears to be healthier than the SS line at high concentrations of NaCl and is supported by the increased gain in fresh weight of the ST (Figure AI-1). Fresh weight of the SS line is significantly reduced ($P<0.01$) even at NaCl concentrations as low as 50 mM whereas in the ST line, fresh weight declined only slightly at the same concentration.

In order to identify proteins whose levels may be altered in the ST line, we subjected the proteins extracted from the SS and ST lines to two-dimensional electrophoresis. Images of two-dimensional electrophoresis gels of protein from ST and SS lines are shown in Figure AI-2. Images of Coomassie blue-stained gels from the SS and ST lines cultured in the absence of NaCl are shown in Figure AI-2A and B, respectively and those stained with silver are shown in Figure AI-2C and D.

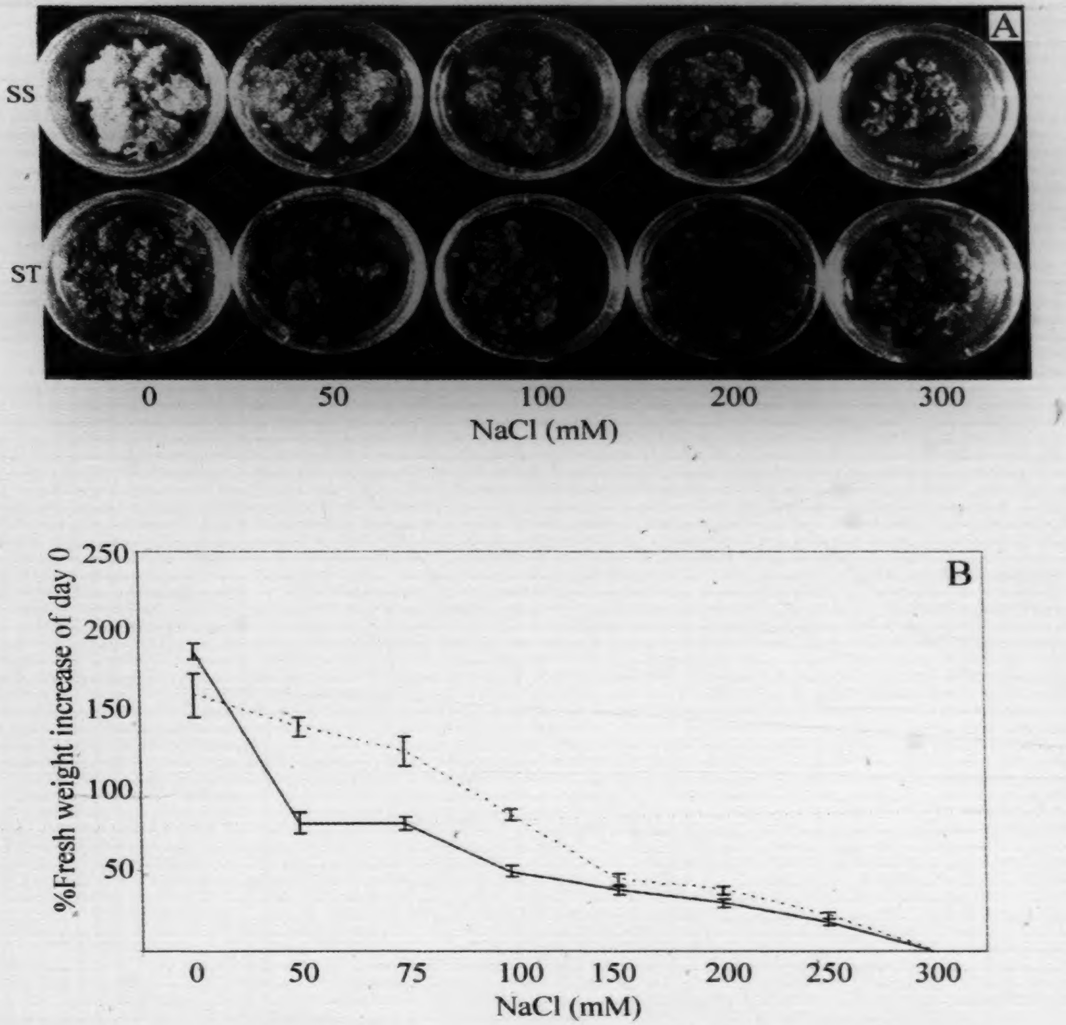
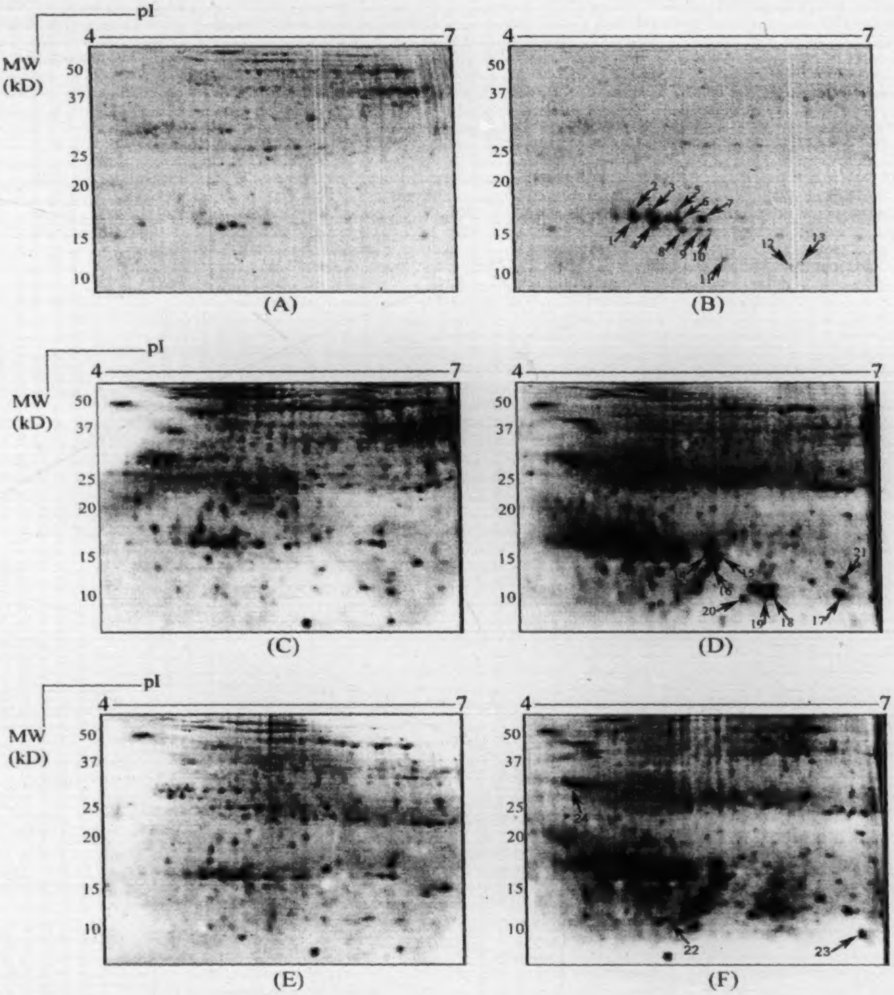


Figure AI-1. Appearance of *Arachis hypogaea* callus lines (A) grown in the presence or absence of NaCl. The letters SS and ST refer to the sensitive and tolerant lines, respectively and the numbers on the Petri dishes refer to the concentration of NaCl in mM. Effects of NaCl on the growth of the SS and ST lines (B). Fresh weights of tissues were measured 12 days after transfer to media containing different concentrations of NaCl and the change in fresh weight was expressed as a percentage of the weight on day 0. Solid and dashed lines represent SS and ST lines, respectively.

A detailed comparison of these images using the Student *t*-test feature of the PDQuest software (Bio-Rad) revealed at least 21 protein spots whose spot volumes were significantly higher ($P<0.05$) or were unique in the ST gels compared to the SS gels (spots 1-13; Figure AI-2A and B; spots 14-21; Figure AI-2C and D. In addition, the silver-stained images of two-dimensional gels obtained with extracts prepared from SS and ST lines cultured in the presence of 200 mM NaCl are shown in Figure AI-2E and F. An analysis of these gels revealed an increase in the levels of at least 3 proteins (Figure AI-2F; spots 22-24). The MASCOT scores for most of the identified spots were higher than the threshold scores (Table AI-1) which is indicative of extensive identities between the MS fingerprint and the sequence in the databases and, that the observed match is not a random event.

The 24 spots (Table AI-1) were excised from the two-dimensional gels and identified using tandem MS. Remarkably, a large number of spots (1-15, 17-19, 21-22; Table AI-1) generated peptide mass fingerprints that identified them as proteins belonging to the PR 10 protein family such as the Ara h 8 allergen. Other proteins identified as being elevated in the ST line in response to salinity included an RNA-binding protein (spot 23), and a 14-3-3 protein (spot 24).


pI
MW (kD)
4
7
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(A)
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Figure AI-2. Two-dimensional electrophoresis of protein extracts prepared from the callus of SS and ST lines of *Arachis hypogaea.* Two-dimensional gels stained from the SS and ST lines cultured in the absence of NaCl and stained with Coomassie blue are shown in (A) and (B), respectively whereas those stained with silver from SS and ST lines are shown in (C) and (D), respectively. Panels (E) and (F) represent silver-stained gels of protein extracts from callus cells cultured in the presence of 200 mM NaCl from the SS and ST lines, respectively. The arrows indicate spots which were identified using tandem Mass Spectrometry.

Table AI-1. Details of proteins identified by ESI-Q-TOF

Spot No.	MS/MS (ESI-Q-TOF)		Identity	§Accession No.	¶Score (Threshold)	Mr/pI	†Fold change
	[*]PM/%	Sequence					
1	3/23%	SVEIVEGNGGPGTIK LTIVEDGETK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	198 (>47)	16942/ 5.03	162 ± 68.45
2	3/23%	SVEIVEGNGGPGTIK LTIVEDGETK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	198 (>48)	16942/ 5.03	73.09 ± 32.6
3	2/20%	GVFTFEDEITSTVPPAK SVEIVEGNGGPGTIK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	80 (>47)	16942/ 5.03	U
4	3/23%	SVEIVEGNGGPGTIK LTIVEDGETK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	230 (>48)	16942/ 5.03	3.92 ± 0.25
5	1/7%	LVEGANGGSIGK	PR10.2C protein [*Lupinus luteus*]	gi|12958727	75 (>48)	16802/ 4.81	U
6	2/20%	GVFTFEDEITSTVPPAK SVEIVEGNGGPGTIK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	74 (>47)	16942/ 5.03	12.17 ± 2.26
7	4/25%	GVHTFEEESTSPVPPAK GVHTFEEESTSPVPPAK LIPAIQSIEIVEGNGGPGTVK LIPAIQSIEIVEGNGGPGTVK	PR10 protein [*Arachis hypogaea*]	gi|52547774	78 (>47)	16208/ 5.34	10.73 ± 0.84
8	5/31%	SVEIVEGNGGPGTIK LTIVEDGETK LVEGPNGGSIGK LVEGPNGGSIGK GDAKPDEEELKK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	231 (>48)	16942/ 5.03	443.01 ± 93.06
9	4/23%	SVEIVEGNGGPGTIK SVEIVEGNGGPGTIK LTIVEDGETK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	201 (>48)	16942/ 5.03	U

10	4/28%	GVFTFEDEITSTVPPAK GVFTFEDEITSTVPPAK SVEIVEGNGGPGTIK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	143 (>48)	16942/ 5.03	U
11	2/17%	SVEIVEGNGGPGTIK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	124 (>47)	16942/ 5.03	211.9 ± 26.43
12	1/9%	SVEIVEGNGGPGTIK	Pathogenesis-related protein 1 (PvPR1) [*Phaseolus vulgaris*]	gi|21044	48 (>47)	16518/ 4.83	U
13	1/9%	SVEIVEGNGGPGTIK	Pathogenesis-related protein 1 (PvPR1) [*Phaseolus vulgaris*]	gi|21044	44 (>46)	16518/ 4.83	U
14	2/14%	LTIVEDGETK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	137 (>46)	16942/ 5.03	17.82 ± 5.36
15	2/14%	LTIVEDGETK LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	118 (>47)	16942/ 5.03	U
16	1/1%	ELLENSPK	RNA polymerase sigma factor [*Bacillus subtilis*]	gi|2633716	38 (>49)	16942/ 5.03	U
17	1/7%	LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	92 (>48)	16942/ 5.03	U
18	1/7%	LVEGANGGSIGK	PR10.2C protein [*Lupinus luteus*]	gi|12958727	78 (>49)	16802/ 4.81	U

19	1/7%	LVEGANGGSIGK	PR10.2C protein [*Lupinus luteus*]	gi|12958727	81 (>48)	16802/ 4.81	U
20	1/2%	TDLPNVVEK	Alcohol dehydrogenase [*Pisum sativum*]	gi|20639	25 (>48)	41870/ 6.09	U
21	1/7%	LVEGPNGGSIGK	Ara h 8 allergen [*Arachis hypogaea*]	gi|37499626	69 (>48)	16942/ 5.03	U
22	4/16%	DADEIVPK LSILEDGK LSILEDGK GDAALSDAVR	Pathogenesis-related protein 17 [*Pisum sativum*]	S42649	165 (>46)	16619/ 5.07	2.37 ± 0.24
23	1/1%	LELSDIAGR	RNA binding protein-like [*Arabidopsis thaliana*]	gi|9293981	43 (>38)	107590/ 5.67	23.55 ± 19
24	1/4%	EAAESTLAAYK	14-3-3 protein [*Vigna angularis*]	gi|13928452	73 (>48)	29299/ 4.66	3.55 ± 0.65
	1/1%	NQADSVVYQTEK	heat shock protein hsp70 [*Pisum sativum*]	gi|445605	56 (>48)	75521/ 5.22	

* Number of peptides matched/ sequence percentage coverage.

§ Accession number is Mascot search result using NCBI and other databases.

† Spots unique (U) or up-regulated ($P < 0.05$) and their fold change ± SE values.

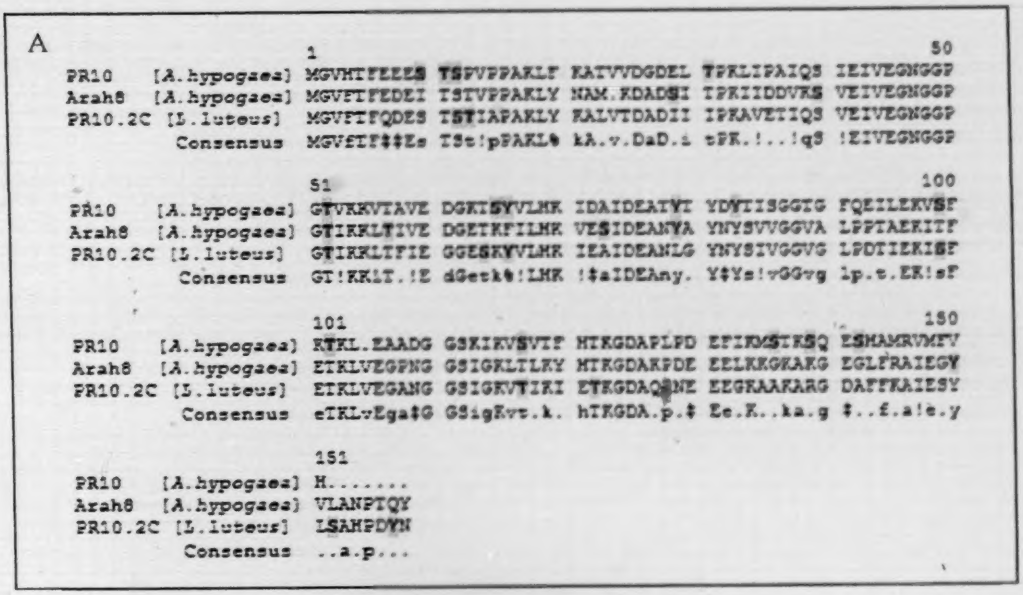
¶ Score is -10 Log (P), where P is the probability that the observed match is a random event. Individual ion scores greater than the threshold value indicate identity or extensive homology ($P < 0.05$).

Due to this abundance of PR 10 proteins in the ST line, we have focused our discussion on this group of proteins although it is possible that the other proteins may also have important roles in mediating salinity tolerance in this line. For example, the 14-3-3 group of proteins has been shown to interact with a number of signaling molecules such as kinases and phosphatases and, may thus play vital roles in mediating important cellular processes in response to environmental changes (Fu *et al.*, 2000).

PR proteins are a group of proteins that are induced in response to both abiotic and biotic stresses and are classified into 14 families based on their biological function or similarities in primary structures (Van Loon and Van Strien, 1999). Among the known families of PR proteins, PR 10 is unique in that they are located in the cytosol and unlike the other families of PR proteins their function is not yet established (Van Loon and Van Strien, 1999). As the name suggests, PR 10 proteins are induced in response to pathogens as well as various abiotic stresses (Van Loon and Van Strien, 1999; Liu *et al.*, 2003; Kav *et al.*, 2004). Furthermore, a recently discovered novel PR 10 has been shown to be specifically induced in the roots of rice plants in response to abiotic stresses via the jasmonate signaling pathway (Hashimoto *et al.*, 2004). The PR 10 family also includes proteins that have allergenic characteristics such as Bet v 1 and Ara h 8 gene products (Mittag *et al.*, 2004).

It was particularly intriguing that most of the proteins identified in this study as being elevated in the ST line belonged to the family of PR 10 proteins. It is possible that the large number of PR 10 proteins, particularly those that were identified as the Ara h 8 proteins are different isoforms of the same gene product and represent differentially phosphorylated states. In order to investigate this hypothesis we have predicted the various potential phosphorylation sites using the Net Phos 2.0 server (Blom *et al.*, 1999). These sites are indicated in Figure AI-3A which also shows the alignment of the amino acid sequences of the Ara h 8 and PR 10 proteins from peanut with a PR 10 protein from lupin. Results from our analysis suggest that there may be several forms of both the peanut PR 10 as well as the Ara h 8 proteins due to phosphorylation at more than one potential site.

Pro-Q Diamond phosphoprotein gel stain is suitable for fluorescent detection of phosphoserine, phosphothreonine- and phosphotyrosine containing proteins in polyacrylamide gels (Steinberg *et al.*, 2003). Image of a two-dimensional gel of protein extracts from the ST line is shown in Figure AI-3B along with the corresponding region from the Coomassie blue stained gel Figure AI-3C. It is apparent that several spots (1-6, 8, 11) whose identities were established as PR 10 proteins (including Ara h 8; Table AI-1) appear to be phosphorylated. The fact that phosphorylation may be important for the biological activities of PR 10 proteins is further supported by the observation that the activity of a PR 10 protein isolated from hot pepper increases by phosphorylation (Park *et al.*, 2004).



```
A
                             1                                                   50
PR10    [A.hypogaea] MGVHTFEEES TSPVPPAKLF KATVVDGDEL TPKLIPAIQS IEIVEGNGGP
Arah8   [A.hypogaea] MGVFTFEDEI TSTVPPAKLY NAM.KDADSI TPKIIDDVKS VEIVEGNGGP
PR10.2C [L.luteus]   MGVFTFQDES TSTIAPAKLY KALVTDADII IPKAVETIQS VEIVEGNGGP
           Consensus MGVfTF##Es TSt!pPAKL% kA.v.DaD.i tPK.!..!qS !EIVEGNGGP

                     51                                                  100
PR10    [A.hypogaea] GTVKKVTAVE DGKISYVLHK IDAIDEATYI YDYTISGGTG FQEILEKVSF
Arah8   [A.hypogaea] GTIKKLTIVE DGETKFILHK VESIDEANYA YNYSVVGGVA LPPTAEKITF
PR10.2C [L.luteus]   GTIKKLTFIE GGESKYVLHK IEAIDEANLG YNYSIVGGVG LPDTIEKISF
           Consensus GT!KKlT.!E dGetk%!LHK !#aIDEAny. Y#Ys!vGGvg lp.t.EK!sF

                     101                                                 150
PR10    [A.hypogaea] KTKL.EAADG GSKIKVSVTF HTKGDAPLPD EFIKMSTKSQ ESMAMRVMFV
Arah8   [A.hypogaea] ETKLVEGPNG GSIGKLTLKY HTKGDAKPDE EELKKGKAKG EGLFRAIEGY
PR10.2C [L.luteus]   ETKLVEGANG GSIGKVTIKI ETKGDAQSNE EEGKAAKARG DAFFKAIESY
           Consensus eTKLvEga#G GSigKvt.k. hTKGDA.p.# Ee.K..ka.g #..f.a!e.y

                     151
PR10    [A.hypogaea] H.......
Arah8   [A.hypogaea] VLANPTQY
PR10.2C [L.luteus]   LSAHPDYN
           Consensus ..a.p...
```

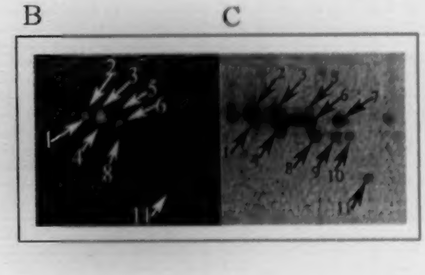


Figure AI-3. (A) Multiple sequence alignment of PR 10 (*Arachis hypogaea)*, Ara h 8 (*Arachis hypogaea*) and PR 10.2C (*Lupin luteus*) are shown and potential phosphorylation sites serine (S), threonine (T) and tyrosine (Y) are highlighted. Multiple sequence alignment was performed using MultAlin software (Corpet, 1988) and prediction of potential phosphorylation sites by Net Phos 2.0 Server (Blom *et al.*, 1999). (B) A section of two-dimensional gel of protein from ST lines stained with Pro-Q Diamond showing the phosphorylation of many spots identified as PR 10 proteins and, (C) the corresponding region from a Coomassie stained gel.

In order to verify whether the proteins identified by Pro-Q Diamond phosphoprotein stain are indeed phosphorylated, we subjected spots 1, 4, 7 and 8 (which were most abundant) once again to MS/MS analysis and searched the non-redundant NCBI database after including phosphorylation as an additional search criterion. These searches revealed that the first two significant hits for spots 1 and 4 were the Ara h 8 protein followed by a PR 10 protein from *Phaseolus vulgaris*. One of the peptides, AVDSFKSVEIVEGNGGPGTIK was identified as being phosphorylated by MASCOT and for spot 8, the second significant hit was a PR 10 protein from *Vigna unguiculata* where the same peptide was identified as being phosphorylated. This peptide corresponds to a highly conserved region among all PR 10 proteins characterized thus far and has been predicted to possess two potential phosphorylation sites (Figure AI-3A). Although we observed the same phosphorylated peptide for spot 7 as well, the score obtained was not significant for it to be a strong hit. Additional experiments, however, are required to unequivocally demonstrate phosphorylation of *A. hypogaea* PR 10 proteins and its role in their biological function(s).

Although there is a strong correlation between the expression of various PR 10 proteins and plant cell responses to stress, their intracellular function remains unknown. PR 10 proteins from some sources have been shown to possess RNase activity (Moiseyev *et al.*, 1994), which may be involved in defense against pathogens (Park *et al.*, 2004). Another plausible function for PR 10 proteins may be in ligand-mediated signaling processes in response to plant stress as evidenced by the ability of many PR 10 proteins to bind ligands including cytokinins, fatty acids, flavonoids

(Fujimoto *et al.*, 1998; Mogensen *et al.*, 2002) and brassinosteroids (Markovic-Housely *et al.*, 2003). Despite the controversy surrounding the physiological activities of PR 10 proteins *in planta*, an emerging role for this protein in mediating plant stress tolerance is becoming increasingly clear. In fact, we have previously demonstrated that the constitutive expression of a pea *PR 10* gene in *B. napus* enhances their germination and early seedling growth suggesting a role in salinity tolerance (Srivastava *et al.*, 2004).

AI.4. Conclusion

The results from this current study indicate that the role of PR 10 proteins in ameliorating abiotic stress responses, in particular salinity, may be conserved in more than one species as evidenced by a clear increase in a number of PR 10 proteins in the ST line of groundnut callus. Additional studies aimed at characterizing the biochemical activity of the peanut PR 10 proteins and the role of phosphorylation is currently underway in our laboratory. Our current findings may lead to the development of novel biochemical markers for selecting salinity-tolerant plants as well as in the engineering of salinity-tolerant crops.

AI.5. References

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis* Science **285:** 1256–1258.

Blom, N., Gammeltoft, S. and Brunak, S. (1999) Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol **294:** 1351–1362.

Boyer, J.S. (1982) Plant productivity and environment Science **218:** 443–448.

Corpet F. (1988) Multiple sequence alignment with hierarchical clustering. Nucl Acids Res **16:** 10881–10890.

Fu H., Subramanian R.R. and Masters S.C. (2000) 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol **40:** 617–647.

Hasegawa, P.M., Bressan, R.A., Zhu, J-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Physiol. Plant Mol Biol **51:** 463–499.

Hashimoto, M., Kisseleva, L., Sawa, S., Furukawa, T., Komatsu, S. and Koshiba, T. (2004) Novel rice PR 10 protein, RSOsPR 10, specifically induced in roots by biotic and abiotic stresses, possibly via the jasmonic acid signaling pathway. Plant Cell Physiol **45:** 550–559.

Jain, M., Mathur, G., Koul, S. and Sarin, N.B. (2001) Ameliorative effects of proline on salt stress-induced lipid peroxidation in cell lines of groundnut (*Arachis hypogaea* L.). Plant Cell Rep **20:** 463–468.

Kav, N.N.V., Srivastava, S., Goonewardene, L. and Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity. Ann Appl Biol **145:** 217–230.

Liu, J-J., Ekramoddoullah, A.K.M. and Yu, X. (2003) Differential expression of multiple PR 10 proteins in western white pine following wounding, fungal infection and cold-hardening. Physiol Plant **119:** 544–553.

Markovic-Housely, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O. and Breitender, H. (2003) Crystal structure of a hypoallergenic isoforms of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J Mol Biol **325:** 123–133.

Mittag, D. Akkerdaas, J., Ballmer-Weber, B.K., Vogel, L., Wensing, M., Becker, W.M., Koppelman, S.J., Knulst, A.C., Helbling, A., Hefle, S.L., van Ree, R. and Vieths, S. (2004) Ara H 8 a Bet v 1 homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. J Allergy Clin Immunol **114:** 1410–1417.

Mogensen, J.E., Wimmer, R., Larsen, J.N. and Spangfort, M.D. (2002) The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J Biol Chem **277:** 684–692.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. and Yakovlev, G.I. (1994) High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta **193:** 470–472.

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497.

Murota, K., Ohshita, Y., Watanabe, A., Aso, S., Sato, F. and Yamada, Y. (1994) Changes related to salt tolerance in thylakoid membrane of photoautotrophically cultured green tobacco cells. Plant Cell Physiol **35:** 107–113.

Nagaoka S. and Takano T. (2003) Salt tolerance-related protein STO binds to a Myb transcription factor homologue and confers salt tolerance in *Arabidopsis* J Exp Bot **54:** 2231–2237.

Park, C.J., Kim, K.J., Shin, R., Park, J.M., Shin, Y.C. and Paek, K.H. (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J **37:** 186–198.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B. and Bennett, J. (2002) Proteomic analysis of rice leaves during drought stress and recovery. Proteomics **2:** 1131–1145.

Singh, K.N., Handa, A.K., Hasegawa, P.M. and Bressan, R.A. (1985) Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiol **79:** 126–137.

Steinberg, T.H., Agnew, B.J., Gee, K.R., Leung, W.Y., Goodman, T., Schulenberg, B., Hendrickson, J., Beechem, J.M., Haugland, R.P. and Patton, W.F. (2003) Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology. Proteomics **3:** 1128–1144.

Srivastava, S., Fristensky, B. and Kav N.N.V. (2004) Constitutive expression of a PR 10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol **45:** 1320-1324.

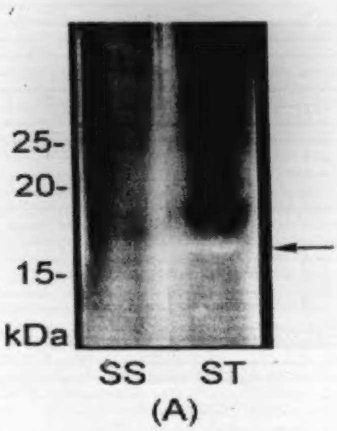
Subramanian, B., Bansal, V.K. and Kav, N.N.V. (2005) Proteome-level investigation of *Brassica carinata*-derived resistance to *Leptosphaeria maculans*. J Agric Food Chem **53:** 313–324.

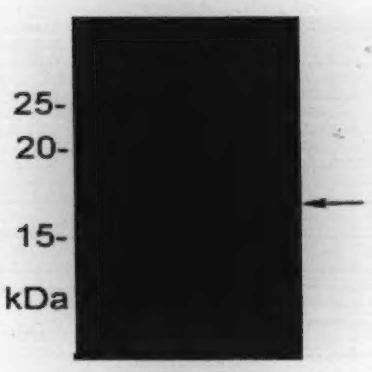
Van Loon, L.C. and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities and comparative analysis of PR1 proteins, Physiol Mol Plant Pathol **55:** 85–97.

Wang, W., Vinocur, B. and Altman, A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218:** 1–14.

Zhu, J-K. (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53:** 247–273.

Appendix II





(B)

Figure AII-1. In-gel RNase activity assay (A) *A. hypogaea* salinity-sensitive (SS) and salinity-tolerant (ST) lines. Arrow points to clear band that is produced as a result of RNase activity in ST line (B) Recombinant Pm PR 10 protein. Arrow points to the region where white band is expected and which corresponds to the molecular weight of PR 10 protein.

Appendix III

Table AIII-1. Endogenous concentration of hormones in 2-week-old *A.thaliana* plants grown in soil.

Hormone	**Quantity ng/g DW^{-1} (n = 3)**			
	Wild-type (Mean ± SE)	**Transgenic 6.9** (Mean ± SE)	**Transgenic 14.9** (Mean ± SE)	**Transgenic 25.20** (Mean ± SE)
IAA	138 ± 10.8	101.4 ± 2.4	130.4 ± 9.1	112.17 ± 9.2
GA1	Not detected			
GA4	6.6 ± 1.3	3.9 ± 0.6	8.2 ± 1.5	4.4 ± .3
ABA	Not detected			